

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | |
|---|---|---|
| (51) International Patent Classification 5 : A61K 35/14, 37/10, C07H 15/12C07H 17/00, C07K 3/00, 13/00 15/00, 17/00, C12N 01/20, C12N 05/00, 05/02, 15/00, C12P 21/06, C12Q 1/00 | A1 | (11) International Publication Number: WO 93/11778 (43) International Publication Date: 24 June 1993 (24.06.93) |
| (21) International Application Number: PCT/US92/10947 (22) International Filing Date: 11 December 1992 (11.12.92) (30) Priority data: 806,709 12 December 1991 (12.12.91) US (60) Parent Application or Grant (63) Related by Continuation US 806,709 (CON) Filed on 12 December 1991 (12.12.91) (71) Applicant (for all designated States except US): THE SCRIPPS RESEARCH INSTITUTE [US/US]; 10666 North Torrey Pines Road, La Jolla, CA 92037 (US). | (72) Inventors; and (75) Inventors/Applicants (for US only) : RUGGERI, Zaverio, M. [IT/US]; 6633 Avenida de Las Pescas, La Jolla, CA 92037 (US). WARE, Jerry, L. [US/US]; 2119 Summer- hill Drive, Encinitas, CA 92024 (US). DE MARCO, Lui- gi [IT/IT]; Via Gemonia, 5, I-33170 Pordenone (IT). MAZZUCATO, Mario [IT/IT]; Via A. Boito, 9, I-30026 Portogruaro (IT). (74) Agents: BARRON, Alexis et al.; Synnestvedt & Lechner, 2600 One Reading Center, 1101 Market Street, Philadel- phia, PA 19107 (US). (81) Designated States: AT, AU, BB, BG, BR, CA, CH, CS, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG). Published With international search report. | |
| (54) Title: BIFUNCTIONAL ANTITHROMBOTIC MOLECULES AND ANTITHROMBOTIC POLYPEPTIDES (57) Abstract A bifunctional molecule is provided that is capable of inhibiting activation of a platelet and/or adhesion of a platelet to a damaged or diseased vascular domain and is capable of inhibiting the binding of thrombins to platelet glycoprotein Ibalpha and the second domain is capable of inhibiting the binding of von Willebrand factor to platelet glycoprotein Ibalpha. Also, a therapeutic composition containing the bifunctional molecule is provided. The process of producing the bifunctional molecule is by culturing a host organism transformed with a biologically functional expression plasmid that contains a DNA sequence encoding the bifunctional molecule under conditions which effect expression of the molecule by the host organism, and recovering the molecule from it. | | |

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|----|--------------------------|----|---------------------------------------|----|--------------------------|
| AT | Austria | FR | France | MR | Mauritania |
| AU | Australia | GA | Gabon | MW | Malawi |
| BB | Barbados | GB | United Kingdom | NL | Netherlands |
| BE | Belgium | GN | Guinea | NO | Norway |
| BF | Burkina Faso | GR | Greece | NZ | New Zealand |
| BG | Bulgaria | HU | Hungary | PL | Poland |
| BJ | Benin | IE | Ireland | PT | Portugal |
| BR | Brazil | IT | Italy | RO | Romania |
| CA | Canada | JP | Japan | RU | Russian Federation |
| CF | Central African Republic | KP | Democratic People's Republic of Korea | SD | Sudan |
| CG | Congo | KR | Republic of Korea | SE | Sweden |
| CH | Switzerland | KZ | Kazakhstan | SK | Slovak Republic |
| CI | Côte d'Ivoire | LI | Liechtenstein | SN | Senegal |
| CM | Cameroon | LK | Sri Lanka | SU | Soviet Union |
| CS | Czechoslovakia | LU | Luxembourg | TD | Chad |
| CZ | Czech Republic | MC | Monaco | TG | Togo |
| DE | Germany | MG | Madagascar | UA | Ukraine |
| DK | Denmark | ML | Mali | US | United States of America |
| ES | Spain | MN | Mongolia | VN | Viet Nam |
| FI | Finland | | | | |

BIFUNCTIONAL ANTITHROMBOTIC MOLECULES
AND ANTITHROMBOTIC POLYPEPTIDES

Cross-Reference to Related Applications

This application is a continuation-in-part of
5 application Serial No. 07/613,004, filed November 13, 1990,
which is a continuation-in-part application Serial No.
07/600,183, filed October 17, 1990, which is a continuation-
in-part of Serial No. 07/519,606, filed May 7, 1990, which is
a continuation-in-part of Serial No. 07/270,488, filed
10 November 4, 1988, now abandoned, which is a continuation of
Serial No. 869,188, filed May 30, 1986, now abandoned. This
application is also a continuation-in-part of application
Serial No. 07/613,083, filed November 14, 1990, which is a
continuation-in-part of Serial No. 07/460,674, filed on
15 January 4, 1990, which is a continuation-in-part of Serial
No. 07/121,454, filed on November 17, 1987, now abandoned.

Field of the Invention

This invention relates to a bifunctional molecule and to
a polypeptide, each of which is useful in the treatment of
20 vascular disorders such as thrombosis. This invention
further relates to the production by recombinant DNA-directed
methods of pharmacologically useful quantities of the
polypeptides of the present invention.

The term "hemostasis" refers to those processes which
25 comprise the defense mechanisms of the body against loss of
circulating blood caused by vascular injury. Processes which

are normal as a physiologic response to vascular injury may lead in pathologic circumstances, such as in a patient afflicted with atherosclerotic vascular disease or chronic congestive heart failure, to the formation of undesired thrombi (clots) with resultant vascular occlusion. Impairment of blood flow to organs under such circumstances may lead to severe pathologic states, including myocardial infarction, a leading cause of mortality in developed countries.

10 The restriction or termination of the flow of blood within the circulatory system in response to a wound or as a result of a vascular disease state involves a complex series of reactions which can be divided into two processes, primary and secondary hemostasis. Primary hemostasis refers to the process of platelet plug or soft clot formation. The platelets are non-nucleated discoid structures approximately 2-5 microns in diameter derived from megakaryocytic cells. Effective primary hemostasis is accomplished by platelet adhesion, the interaction of platelets with the surface of damaged vascular endothelium on which are exposed underlying collagen fibers and/or other adhesive macromolecules such as proteoglycans and glycosaminoglycans to which platelets bind.

20 Secondary hemostasis involves the reinforcement or crosslinking of the soft platelet clot. This secondary process is initiated by proteins circulating in the plasma (coagulation factors) which are activated during primary hemostasis, either in response to a wound or a vascular disease state. The activation of these factors results ultimately in the production of a polymeric matrix of the protein fibrinogen (then called fibrin) which reinforces the soft clot. The conversion of fibrinogen to fibrin is catalyzed by thrombin, one of the coagulation factors. As described below, thrombin also participates in the reactions which activate platelets.

30 Therapeutic drugs for controlling thrombosis have been classified according to the stage of hemostasis which is affected by the administration thereof. Such prior art compositions are typically classified as anticoagulants, thrombolytics and platelet inhibitors.

- The anticoagulant therapeutics typically represent a class of drugs which intervene in secondary hemostasis. Anticoagulants typically have no direct effect on an established thrombus, nor do they reverse tissue damage.
- 5 Associated with the use of existing anticoagulants is the hazard of hemorrhage, which may under some conditions be greater than the clinical benefits otherwise provided by the use thereof. As a result, anticoagulant therapy must be closely monitored. Certain anticoagulants act by inhibiting
- 10 the synthesis of vitamin K-dependent coagulation factors resulting in the sequential depression of, for example, factors II, VII, IX, and X. Representative anticoagulants which are used clinically include coumarin, dicoumarol, phenindione, and phenprocoumon.
- 15 Thrombolytics act by lysing thrombi after they have been formed. Thrombolytics such as streptokinase and urokinase have been indicated for the management of acute myocardial infarctions and have been used successfully to remove intravascular clots if administered soon after thrombosis
- 20 occurs. However, the lysis effected thereby may be incomplete and nonspecific, i.e., useful plasma fibrinogen, in addition to fibrin polymers within clots, is affected. As a result, a common adverse reaction associated with the use of such therapeutics is hemorrhage.
- 25 A third classification, antiplatelet drugs, includes drugs which suppress primary hemostasis by altering platelets or their interaction with other circulatory system components. The present invention relates to this classification of antiplatelet drugs.

30 Reported Developments

- Specific antiplatelet drugs operate by one or several mechanisms. A first example involves reducing the availability of ionized calcium within the platelet cytoplasm thereby impairing activation of the platelet and resultant
- 35 aggregation. Pharmaceuticals representative of this strategy include prostacyclin, and also Persantine® (dipyridamole) which may affect calcium concentrations by affecting the concentration of cyclic AMP. Numerous side effects related

to the administration of these compounds have been reported. An additional class of antiplatelet drugs acts by inhibiting the synthesis of thromboxane A_2 within the platelet, reducing the platelet activation response. Non-steroidal anti-inflammatory agents, such as ibuprofen, phenylbutazone and naphthroxane may produce a similar effect by competitive inhibition of a particular cyclooxygenase enzyme, which catalyzes the synthesis of a precursor of thromboxane A_2 . A similar therapeutic effect may be derived through the administration of aspirin which has been demonstrated to irreversibly acetylate a cyclooxygenase enzyme necessary to generate thromboxane A_2 . A third anti-platelet mechanism has involved the platelet membrane so as to interfere with surface receptor function. One such drug is dextran, a large branched polysaccharide, which is believed to impair the interaction of fibrinogen with platelet receptors that are exposed during aggregation. Dextran is contraindicated for patients with a history of renal problems or with cardiac impairment. The therapeutic ticlopidine is stated to inhibit platelet adhesion and aggregation by suppressing the binding of von Willebrand factor and/or fibrinogen to their respective receptors on the platelet surface. However, it has been found that ticlopidine possesses insufficient specificity to eliminate the necessity of administering large doses which, in turn, may be associated with clinical side effects.

The aforementioned pharmaceuticals are foreign to the body and may cause numerous adverse clinical side effects, there being no way to prevent such compounds from participating in other aspects of a patient's physiology or biochemistry, particularly if high doses are required. It would be desirable to provide for pharmaceuticals having such specificity for certain of the reactions of hemostasis, that they could be administered to patients at low doses, such doses being much less likely to produce adverse effects in patients.

An example of a pharmaceutical which is representative of a therapeutic that is derived from natural components of the hemostatic process is described in EPO Publication No.

317278. This publication discloses a method for inhibiting thrombosis in a patient by administering to the patient a therapeutic polypeptide comprised of the amino-terminal region of the α chain of platelet membrane glycoprotein Ib, or a subfragment thereof.

The present invention is directed to the provision of a therapeutic molecule having a plurality of antithrombotic functions which are based on natural components of the hemostatic mechanism.

10 Summary of the Invention

Broadly stated, this invention provides for an antithrombotic molecule which is capable of inhibiting the adhesion of platelets to damaged or diseased vascular surfaces and which is capable of inhibiting the activation or aggregation of platelets. The antithrombotic molecule of the present invention is polyfunctional in that the molecule includes a plurality of functional groups of a nature such that the functional groups are capable of interfering with a plurality of materials which are normally involved in the body process of clot formation. One aspect of the present invention comprises a bifunctional molecule which has the capability of affecting two processes in the formation of a thrombus, namely (1) the interaction of thrombins with glycoprotein Ib α receptor of platelets, and (2) interaction of von Willebrand factor with glycoprotein Ib α receptor of platelets.

One aspect of the present invention is the provision of a bifunctional molecule which is capable of inhibiting activation of a platelet and/or adhesion of a platelet to a damaged or diseased vascular surface and which comprises two linked domains, a first domain capable of inhibiting the binding of thrombins to platelet glycoprotein Ib α and a second domain capable of inhibiting the binding of von Willebrand factor to platelet glycoprotein Ib α .

35 Examples of structures which comprise one or both of the linked domains of the bifunctional molecule of this invention are antibodies or active fragments thereof, polypeptide fragments derived from proteins that participate in

hemostasis, and organic analogs patterned on the aforementioned structures and having the binding activity thereof. Accordingly, the present invention provides also a bifunctional molecule having a first domain selected from the

5 group consisting of:

- (A) an antibody, or an active fragment thereof, having as its epitope, or as a part thereof, all or part of a thrombin binding site of the platelet provided by glycoprotein Ib α ;
- 10 (B) a polypeptide comprising approximately the amino terminal His¹ to Arg²⁹³ fragment of glycoprotein Ib α , in derivatized or underivatized form, or one or more subfragments thereof containing all or part of the thrombin-binding site thereof;
- 15 (C) a mutant or derivative of a polypeptide of (B) above; and
- (D) an organic analog patterned on any of (A) to (C) above and having all or part of the binding activity thereof;

20 and a second domain selected from the group consisting of:

- (A) an antibody, or an active fragment thereof, having as its epitope, or as a part thereof, all or part of the von Willebrand factor binding site of glycoprotein Ib α ;
- 25 (B) a polypeptide comprising approximately the Arg⁴⁴¹ to Val⁷³³ fragment of mature von Willebrand factor subunit, in derivatized or underivatized form, or one or more subfragments thereof containing all or part of the glycoprotein Ib α binding site thereof;
- 30 (C) a mutant or derivative of a polypeptide of (B) above; and
- (D) an organic analog patterned on any of (A) to (C) above and having all or part of the binding activity thereof.

35 A particularly preferred embodiment of a bifunctional molecule having antithrombotic activity is represented by a first domain comprising a fragment of LJ-Ib10 antibody containing all or part of the variable region thereof and a second domain comprising that fragment of mature von

Willebrand factor subunit from approximately residue Arg⁴⁴¹ to approximately residue Val⁷³³ thereof, or a subfragment thereof containing residues Leu⁶⁹⁴ to Pro⁷⁰⁸.

Another aspect of the present invention is based upon
5 the discovery that certain of the tyrosine residues of the amino terminal region of platelet glycoprotein Iba as determined from humans are sulfated thereby facilitating, for example, interaction with thrombins. Peptides or polypeptides containing such sulfated tyrosines have utility
10 as antithrombotics. Accordingly, there is provided a polypeptide comprising approximately the amino terminal His¹ to Arg²⁹³ fragment of glycoprotein Iba, in derivatized or underivatized form, or one or more subfragments thereof, said polypeptide containing also a sulfate group attached to the
15 phenolic oxygen of one or more tyrosine residues of said polypeptide.

The invention also provides for DNA sequences, expression plasmids and recombinant host cells through which may be expressed numerous of the above-described bifunctional
20 molecules and polypeptides.

Although the invention is described initially in terms of inhibiting the binding of thrombins and/or von Willebrand factor to platelets (cell-like structures that participate in clotting and that are derived from cells of megakaryocytic
25 lineage), it is recognized that GPIba (or encoding mRNA therefor) has been detected in cells other than megakaryocytic cells, for example, cells of the vascular endothelium. This invention provides, therefore, additional strategies for antithrombotic therapy. Accordingly, there is
30 also provided a bifunctional molecule which is capable of inhibiting activation of a cell and which comprises two linked domains, a first domain capable of inhibiting the binding of thrombins to glycoprotein Iba of a cell and a second domain capable of inhibiting the binding of von
35 Willebrand factor to glycoprotein Iba of a cell.

A further additional strategy for antithrombotic therapy comprises a method for inhibiting activation of endothelial cells, or smooth muscle cells, in a patient, said method comprising administering to said patient an effective amount

of a therapeutic composition comprising the amino terminal His¹ to Arg²⁹³ fragment of GPIIb α , in derivatized or underivatized form, or one or more subfragments thereof, and a pharmaceutically acceptable carrier.

5

Brief Description of the Drawings

Figure 1 shows the structure of exemplary peptides, derived from glycoprotein Iba α , that inhibit binding of thrombins to platelets.

Figure 2 shows the effect of an unsulfated synthetic peptide, corresponding to residues 265-285 of glycoprotein Iba α , on the binding of thrombins to platelets.

10

Definitions

Unless indicated otherwise herein, the following terms have the indicated meanings.

15 Transcribed Strand - a DNA strand whose nucleotide sequence is read 3' \rightarrow 5' by RNA polymerase to produce mRNA, being also referred to as the "noncoding strand".

Coding Strand or Non-Transcribed Strand - a strand which is the antiparallel complement of the transcribed strand and
20 which has a base sequence identical to that of the mRNA produced from the transcribed strand except that thymine bases are present (instead of uracil bases of the mRNA), and referred to as "coding" because like mRNA, and when examined 5' \rightarrow 3', the codons for translation may be directly
25 discerned.

Reducing Conditions - refers to the presence of a "reducing" agent in a solution containing von Willebrand factor, or polypeptides derived therefrom, which agent causes the disruption of disulfide bonds of the VWF.

30 Viral Expression Vector - a vector similar to an expression plasmid except that the DNA may be packaged into a viral particle that can transfect cells through a natural biological process.

Downstream - a nucleotide of a transcribed strand of a
35 structural gene is said to be downstream from another section of the gene if the nucleotide is normally read by RNA polymerase after the earlier section of the gene; the

complimentary nucleotide of the nontranscribed strand, or the corresponding base pair within the double stranded form of the DNA, are also denominated downstream.

5 Additionally, and making reference to the direction of transcription and of translation within the structural gene, a restriction endonuclease sequence added upstream (or 5') to the gene means it is added before the sequence encoding the amino terminal end of the protein, while a modification created downstream (or 3') to the structural gene means that
10 it is beyond the carboxy terminus-encoding region thereof. Derivatized Polypeptide - a polypeptide which comprises an amino acid sequence that contains also additional groups, such as, but not limited to, sulfate, esters, additional amino acid sequence, glycosyl groups, or glycosylation as may
15 be added to the polypeptide by posttranslational modification in cells, or as may be added artificially; ("underivatized polypeptide" is one consisting solely of an amino acid sequence); for example, GPIb α and vWF, as produced in mammalian cells, are typically glycosylated and therefore
20 derivatized, but underivatized forms thereof are also useful in the practice of the invention.

Mature vWF - circulating vWF as found in the plasma or as bound to the subendothelium, and consisting of a population of polypeptide monomers which are typically associated into
25 numerous species of multimers (up to about 30 subunits) thereof, each subunit being 2,050 residues in length; when expressed in mammalian cells, mature vWF is usually glycosylated.

Thrombins - those forms of thrombin which, at physiologically
30 relevant concentrations, bind to glycoprotein Ib α of platelets or cells, such binding (interaction) causing in whole or part, directly or indirectly, platelet (or cell) activation; α -thrombin is such a form of thrombin whereas γ -thrombin is not; β -, ϵ - and ζ -thrombins, as those molecules
35 are known in the art, are examples of thrombins within the definition used herein.

In addition, thrombin exists in many other forms, in such forms being within the definition herein if their behavior is consistent with the above-identified functional

criteria. The term "thrombin", as used herein, refers to all forms of thrombin, including "thrombins".

Biological Activity - one or more functions, effects of, activities performed or caused by a molecule in a biological context (that is, in an organism or in an in vitro facsimile); a characteristic biological activity of the 52/48 kDa monomeric fragment of the mature von Willebrand factor subunit is the potential ability to bind to only one platelet GPIIb/IIIa receptor thereby enabling the molecule to inhibit modulator (such as botrocetin) or stimulus-induced binding of multimeric vWF to platelets; other resultant or related effects of the undimerized 52/48 kDa species include inhibition of platelet activation, aggregation, and also adhesion to surfaces, and the inhibition of thrombosis; a characteristic biological activity of "thrombins" is binding to high affinity sites on platelets in glycoprotein IIb/IIIa, said binding being associated with platelet activation; an additional activity of thrombins is acting as an enzyme to convert fibrinogen into fibrin monomers.

Table 1 shows the standard three letter designations for amino acids as used in the present application.

TABLE I

| | | |
|----|---------------|-----|
| | Alanine | Ala |
| | Cysteine | Cys |
| 25 | Aspartic Acid | Asp |
| | Glutamic Acid | Glu |
| | Phenylalanine | Phe |
| | Glycine | Gly |
| | Histidine | His |
| 30 | Isoleucine | Ile |
| | Lysine | Lys |
| | Leucine | Leu |
| | Methionine | Met |
| | Asparagine | Asn |
| 35 | Proline | Pro |
| | Glutamine | Gln |
| | Arginine | Arg |
| | Serine | Ser |
| | Threonine | Thr |
| 40 | Valine | Val |
| | Tryptophan | Trp |
| | Tyrosine | Tyr |

Detailed Description of the Invention

A "Sequence Listing" pursuant to 37 CFR §1.821(c) for nucleotide and amino acid sequences disclosed or referred to herein is appended and made part of this application.

5 The terms "peptide" and "polypeptide" are used herein interchangeably.

With respect to the provision of the bifunctional molecules and polypeptides of the invention the following should be noted.

10 It is preferred to use human amino acid and nucleic acid sequences for the production of the bifunctional molecules and polypeptides. However, it is within the scope of the invention to so utilize the sequences of other mammals, in particular where the amino acid or nucleic acid sequence
15 domain of interest possesses no mutations (in comparison with the human sequence) or possesses mutations, provided that they do not cause adverse effects, for example, initiate an immune response in a patient that cannot be safely controlled when the resultant product is administered in otherwise
20 therapeutic doses.

The present invention encompasses also the use of bifunctional molecules or polypeptides (and/or the encoding human DNA sequences therefor) that contain, relative to the normal human sequence, mutations which do not affect
25 significantly the biological activity thereof. As elaborated below, it is also within the scope of the invention to prepare modified forms of the bifunctional molecules and polypeptides of the invention such as by mutation of an encoding DNA sequence therefor or by derivitization thereof
30 (for example, sulfation, glycosylation, esterification, etc.), by rearranging the domains thereof, or by providing a biologically active fragment of a bifunctional molecule or polypeptide of the invention. Furthermore, it is possible that there are minor biologically unimportant differences
35 between the actual DNAs and polypeptides manipulated or otherwise utilized in the practice of the invention and the structural sequences of amino acids or nucleotides thereof as reported herein. It is understood that the invention encompasses also any such biologically unimportant

variations.

Introduction

The designs of the bifunctional molecules and of the polypeptides of the present invention are based upon the interaction of each of the proteins von Willebrand factor and thrombins with glycoprotein Iba receptors of platelets or cells. For background purposes, there is set forth hereafter information concerning these macromolecules and their role in hemostasis and thrombosis.

10 Background Information - vWF, GPIba and Thrombin

von Willebrand factor ("vWF") performs an essential role in normal hemostasis during vascular injury and is also of central importance in the pathogenesis of acute thrombotic occlusions in diseased blood vessels. Both of these roles involve the interaction of vWF with platelets which are induced to bind at the affected site and are then crosslinked. It is believed that single platelets first adhere to a thrombogenic surface after which they become activated, a process involving major metabolic changes and significant morphological changes within the platelet. Activation is evidenced by the discharge of platelet storage granules containing adhesive substances such as additional vWF (an adhesive protein), secretion of calcium ions, and the expression on the surface of the platelet of additional functional adhesive sites. Once activated, and as a part of normal hemostasis, platelet cells become aggregated, a process which involves extensive crosslinking of the platelets with additional types of adhesive proteins. As described below, platelets are also activated by thrombin.

30 The adhesion of platelets to damaged or diseased vessels occurs through mechanisms that involve specific platelet membrane receptors which interact with specialized adhesive molecules. One such platelet receptor is the glycoprotein Ib-IX complex which consists of a noncovalent association of two integral membrane proteins, glycoprotein Ib (GPIb) and glycoprotein IX (GPIX). By way of background, it is noted that GPIb is a two-chain molecule having an apparent molecular mass of approximately 160 kDa. GPIb is composed of a heavy (alpha, or "GPIba") chain, having a molecular mass of

approximately 145 kDa, linked by disulfide bonds to a light (beta, or GPIb β) chain, having a molecular mass of approximately 22 kDa. GPIb is an integral membrane protein and both the alpha- and beta- chains described above have 5 transmembrane domains.

The adhesive ligand of the GPIb-IX complex is the protein vWF which is found as a component of the subendothelial matrix, as a component of the α -granules secreted by activated platelets, and also as a circulating 10 blood plasma protein. The actual binding site of vWF on the GPIb-IX receptor has been localized on the amino terminal (His¹-Arg²⁹³) region of the α chain of glycoprotein Ib. This region of the polypeptide may be prepared as a fragment of GPIb α having a molecular weight of 45 kDa using, for example, 15 trypsin to effect the necessary proteolytic cleavage.

Certain of the antithrombotic bifunctional molecules and of the polypeptides of the invention are patterned upon or directed to this amino terminal region (45 kDa) of GPIb α .

Inhibition of vWF-GPIb α interaction results in the 20 prevention of primary hemostasis and the induction of an anti-thrombotic state useful in prevention of diseases in which occlusion of blood vessels plays an important role. As described below, inhibition of thrombin-GPIb α interaction is similarly important.

25 vWF exists as a series of high molecular weight multimers of up to 30 glycosylated subunits per multimer in which the subunits are believed to be identical, with each having an approximate molecular weight of 270,000 (270 kDa). Formation of an initial monolayer of platelets covering 30 injured endothelial surfaces is believed to involve a bridging function in which surface bound multimeric vWF binds on the one side to components of the subendothelium, such as collagen or proteoglycans, and on the other side to the GPIb α receptor of a platelet membrane. Evidence that vWF is 35 necessary for thrombus formation has been provided by studies using anti-vWF monoclonal antibodies to induce a deficiency in circulating vWF, Bellinger, D.A. et al., Proc. Natl. Acad. Sci., USA, 84, 8100-8104 (1987), and by studies utilizing a monoclonal antibody specific for the platelet glycoprotein

IIB/IIIA receptor, Collier, B.S. et al., Blood, 68, 783 (1986). The essential role of vWF in hemostasis and thrombosis is further evidenced in patients who are deficient in the glycoprotein Ib-IX complex. These persons exhibit a disease state characterized by severe bleeding following nominal vascular injury under otherwise nonpathologic conditions.

It is believed that the interaction of multimeric vWF with glycoprotein Ib-IX complex (at GPIb(α)) results in platelet activation and facilitates the recruitment of additional platelets to a now growing thrombus. The rapidly accumulating platelets are also crosslinked (aggregated) by the binding of fibrin (produced itself from fibrinogen by the action of thrombin) at platelet glycoprotein IIB-IIIA receptor sites, and also by the binding of vWF at these sites, and/or at additional glycoprotein Ib-IX receptor sites. In addition, the glycoprotein IIB/IIIA receptor may also be involved in the formation of the initial monolayer of platelets. The tetrapeptide Arg·Gly·Asp·Ser (SEQ ID NO: 1) (vWF residues 1744 to 1747), a sequence which vWF shares with many other adhesive proteins, is believed to represent the platelet glycoprotein IIB-IIIA binding site. Of particular importance in the above processes is the multimeric and multivalent character of circulating vWF, which enables the macromolecule to effectively carry out its binding and bridging functions.

Thrombin also performs an essential role in normal hemostasis during vascular injury and is also of central importance in the pathogenesis of acute thrombotic occlusions in diseased blood vessels. Certain of these functions involve the binding (interaction) of thrombin with platelet GPIb α . Interfering with this binding is a central achievement of the present development.

Thrombin, a protein having a molecular weight of about 37,000, is one of the most important components of the hemostasis process, catalyzing or regulating numerous key reactions thereof. The properties of thrombin are reviewed in, for example, Fenton, J.W. et al., Blood Coagulation and Fibrinolysis, 2, 69-75 (1991), Mann, K.G. et al., Annals of

the New York Academy of Sciences, 614, 63-75 (1991).

Briefly, thrombin is a serine protease, produced from proteolysis of its precursor protein, prothrombin. Among thrombin's most important functions related to hemostasis are: (A) catalyzing the conversion of fibrinogen to fibrin monomers which polymerize as a matrix reinforcing soft platelet clots; and (B) activation of platelets. Thrombin also participates in many other cellular processes, including stimulation of smooth muscle contraction, stimulation of endothelial cells to release tissue plasminogen activator, alteration of the permeability of endothelial cell monolayers, and acting as a mitogen toward fibroblasts. Consistent with the complex scope of its functions, thrombin is also known to contain exocites, which are sites in its tertiary structure other than the catalytic site where substrates or inhibitors bind and align for more efficient interaction.

Many of the activities of thrombin must be, or are most efficiently, accomplished on the surface of platelets. In this regard, providing polypeptides that regulate the interaction of thrombin with platelets so as to achieve an antithrombotic effect is a key achievement of the invention.

In connection with inhibiting GPIIb α -thrombin interaction, it is noted also (see, for example, Fenton, J.W. et al.) that there are many forms of thrombin, not all of which are useful in the practice of the invention. The definition section provided herein defines those forms of thrombin (herein "thrombins") which are useful in the practice of the invention.

As described below, a further important aspect of the present development is the recognition that the binding domains of GPIIb α for thrombin and for vWF are in close proximity, making possible the design of bifunctional antithrombotic molecules.

As stated above, these processes are normal as a physiologic response to vascular injury. However, they may lead in pathologic circumstances, such as in diseased vessels, to formation of undesired platelet thrombi with resultant vascular occlusion. Other circumstances in which

it is desirable to prevent deposition of platelets in blood vessels include the prevention and treatment of stroke, and to prevent occlusion of arterial grafts. Platelet thrombus formation during surgical procedures may also interfere with attempts to relieve preexisting vessel obstructions.

Information Concerning the Structure of vWF and GPIb α and the Design of Therapeutics of the Present Invention Based Thereon

von Willebrand Factor

As mentioned above, von Willebrand factor which circulates in the blood exists as a series of high molecular weight multimers containing up to 30 glycosylated subunits per multimer in which the subunits are believed to be identical, each having an approximate molecular weight of about 270 kDa. The circulating "mature" human subunit consists of 2050 amino acid residues. Its structure is the final result of extensive post-translational processing.

The domain of the vWF subunit which binds to the platelet membrane glycoprotein Ib-IX receptor (GPIb α) has been identified within a fragment of vWF. The fragment may be generated by trypsin digestion, followed by disulfide reduction, and extends from approximately residue 449 (valine) of the circulating subunit to approximately residue 728 (lysine) thereof. Current evidence indicates that this segment also contains (between residues 509 and 695 thereof) binding domains for components of the subendothelium, such as collagen and proteoglycans. The primary and tertiary structure of von Willebrand factor and the location of functional domains thereof is reviewed by Titani, K. et al., "Primary Structure of Human von Willebrand Factor" in Coagulation and Bleeding Disorders: The Role of Factor VIII and von Willebrand Factor, T. Zimmerman and Z.M. Ruggeri, eds., Marcel Dekker, New York, 1989.

The above-mentioned residue 449-728 fragment has a molecular weight of about 50 kilo daltons (50 kDa). This 50,000 molecular weight fragment is referred to also as a "52/48" fragment reflecting the fact that human enzyme systems glycosylate the fragment contributing to its molecular weight. The amount of glycosylation varies from molecule to molecule, with two weights, 52,000 and 48,000,

being most common. Without the additional weight contributed by glycosylation, the polypeptide has a molecular weight of approximately 38,000. Very little polymorphism has been reported in the 52/48 human sequence with one significant exception - histidine/aspartic acid at position 709, see Mancuso, D.J. et al. J. Biol. Chem., 264(33), 19514-19527, Table V, (1989).

Polypeptides derived from the above mentioned region of the circulating (mature) von Willebrand factor subunit - from approximately residue 449 to approximately residue 728, or subfragments thereof are considered useful as antithrombotic pharmaceuticals when added to blood in such sufficient amounts as to compete successfully with multimeric vWF for platelet GPIIb/IIIa receptor sites, thereby preventing monolayer formation by, or crosslinking of, the platelets in circumstances where thrombus formation is undesirable, such as in the treatment of vascular disorders.

With respect to the design of domains of bifunctional antithrombotic molecules of the present invention patterned upon vWF, the following information is of particular interest.

A fragment of multimeric von Willebrand factor from the blood having platelet glycoprotein IIb/IIIa binding activity and of approximately 116,000 (116 kDa) molecular weight is isolated by digesting vWF with trypsin. Treatment of the 116 kDa fragment with a reducing agent capable of cleaving disulfide bonds results in the formation of a pair of identical fragments. Each of the identical fragments (which together comprise the 116 kDa polypeptide) has an apparent molecular weight of about 50 kDa. (Polypeptide molecular weight are typically measured by migration, relative to standards, in a denaturing gel electrophoresis system. Weight values which result are only approximate.)

The 52/48 fragment has been demonstrated to competitively inhibit the binding of vWF to platelets. However, manipulation of the 52/48 fragment or its unglycosylated 38 kDa equivalent has proved difficult. Successful manipulation of the fragment (whether derived from blood [Fugimura, Y. et al., J. Biol. Chem., 261(1), 381-385

(1986), and Fugimura, Y. et al., J. Biol. Chem., 262, 1734-1739 (1987)], or expressed from recombinant bacterial host cells, Examples 3 to 6 herein) has typically required that at least certain of the cysteine residues thereof be reduced and permanently alkylated or replaced, through mutation, by other amino acids. Without this treatment, undesired reaction of the cysteine residues thereof invariably occurs, leading to the formation of insoluble and biologically inactive polypeptide aggregates unsuited for effective use as therapeutics.

Factors involved in the design of residue 449-728 vWF fragments which avoid these difficulties are as follows. It is known that the residue 449-728 fragment of mature vWF subunit, which contains the platelet glycoprotein Ib(α) binding domain, has cysteine residues at positions 459, 462, 464, 471, 474, 509 and 695. It is known also that all of the cysteine residues of the mature vWF subunit are involved in disulfide bonds. (Legaz, et al., J. Biol. Chem., 248, 3946-3955 (1973)). Marti, T. et al. Biochemistry, 26, 8099-8109 (1987) conclusively identified mature subunit residues 471 and 474 as being involved in an intrachain disulfide bond. Residues 509 and 695 were identified as being involved in a disulfide bond, although it was not demonstrated whether this pairing was intrachain or interchain (that is, within the same mature vWF subunit). Mohri, H. et al. J. Biol. Chem., 263(34), 17901-17904 (1988) inhibited the ristocetin-induced binding of ^{125}I -labelled multimeric vWF to formalin-fixed platelets with peptide subfragments of the 449-728 subunit fragment. Peptide subfragments fifteen residues in length were synthesized and tested. Those peptides which represent subunit sequence contained within, or overlapping with, two distinct regions Leu⁴⁶⁹ to Asp⁴⁹⁸ and Glu⁶⁸⁹ to Val⁷¹³ were found to be active.

Mohri concluded that the GPIb(α) binding domain of vWF was formed by residues contained in two discontinuous sequences Cys⁴⁷⁴-Pro⁴⁸⁸ and Leu⁶⁹⁴-Pro⁷⁰⁸ maintained in proper conformation in native vWF by disulfide bonding, although the authors were unable to identify the cysteine residue which formed the stabilizing bond(s) and whether the bonds were

intra or interchain. Subsequently, it has been established that residues 509 and 695 form an intrachain disulfide bond (see Examples 3-7 for representative polypeptides, the design of which incorporates this information).

5 Examples of vWF fragments which are suitable as domains in the design of the bifunctional molecules of the invention include:

- 10 (A) a polypeptide comprising reduced and alkylated 52/48 fragment as derived from blood (Fugimura, Y. et al.), or one or more subfragments thereof containing all or part of the GPIb α -binding domain thereof;
- 15 (B) a polypeptide comprising approximately the residue 441 to 733 fragment of vWF subunit expressed from recombinant bacterial host cells (whether or not containing a disulfide bond linking cysteine residues 509 and 695, see Examples 3-5), in derivatized or underivatized form, or a subfragment thereof having GPIb α -binding activity (see, for
- 20 example, representative species described in Example 6);
- 25 (C) a polypeptide comprising approximately the residue 441 to 730 fragment of vWF subunit expressed from recombinant mammalian host cells, as described for example in Example 7, in derivatized or underivatized form, or a subfragment thereof having GPIb α -binding activity.

The above-mentioned polypeptides are the subject of the aforementioned copending '004, '183 and '606 U.S.

30 applications.

Glycoprotein Ib α

A complete cDNA encoding human GPIb α polypeptide has been determined by Lopez et al., Proc. Natl. Acad. Sci. USA, 84, 5615-5617 (1987). The gene for GPIb α has been cloned

35 from a genomic cosmid library utilizing a partial cDNA clone as a probe, and its sequence, including introns, has been determined by Wenger, Biochemical and Biophysical Research Communications, 156(1), 389-395 (1988). The GPIb α sequence predicted thereby consists of a 16 amino acid signal peptide,

Met¹⁶ through Pro¹, followed by a 610 amino acid mature peptide or polypeptide region, His¹ through Leu⁶¹⁰. The nucleotide numbering system of Wenger is followed herein.

The complete sequence of the aforementioned 45 kDa tryptic fragment of GPIb α comprises His¹ through Arg²⁹⁰ or Arg²⁹³. Representative techniques whereby the His¹ to Leu⁶¹⁰ GPIb α polypeptide and also the His¹ to Arg²⁹³ fragment thereof (as a His¹ to Ala³⁰² construct) can be expressed from recombinant mammalian host cells are provided in Example 2.

10 Production of the 45 kDa fragment of GPIb α from blood has been described previously, Vicente, V. et al., J. Biol. Chem., 265(1), 274-280 (1990), see also Handa, M. et al., J. Biol. Chem., 261(27), 12579-12585 (1986). The structure and properties of GPIb α are reviewed in Ruggeri, Z.M., The

15 Platelet Glycoprotein Ib-IX Complex, in Progress in Hemostasis and Thrombosis, vol. 10, p.3568, Collier, B.S. ed., W.B. Saunders Co., Philadelphia, 1991.

Fragments of GPIb α suitable as domains in the design of the bifunctional molecules or polypeptides of the invention

20 include:

- (A) a polypeptide comprising approximately the amino terminal His¹ to Arg²⁹³ fragment of GPIb α in derivatized or underivatized form;
 - (B) a polypeptide comprising one or more subfragments
- 25 of a polypeptide of (A) above, and containing all or part of a binding site for thrombins and/or all or part of a binding site for vWF.

The above-mentioned polypeptides are the subject of the aforementioned copending '083 and '674 U.S. applications.

30 The invention provides for polypeptides capable of inhibiting the binding of thrombins to GPIb α receptor on the surface of platelets or cells, and for bifunctional molecules derived therefrom which are capable of inhibiting the binding of thrombins and/or vWF to said GPIb α receptor. In order

35 that the nature of the bifunctional molecules of the invention be fully appreciated, there is first provided a description of the aforementioned polypeptides capable of inhibiting binding by thrombins.

Polypeptides of the Invention

The practice of the invention includes identification within the amino terminal His¹ to Arg²⁹³ fragment of the GPIb α polypeptide of a high affinity binding site for thrombins, and provision of antithrombotic polypeptides patterned thereon that inhibit binding (interaction) of thrombins at this site. Said polypeptides, whether in derivatized or underivatized form, comprise subfragments (or combinations of subfragments) of the His¹ to Arg²⁹³ fragment of GPIb α and are believed to function by competing with platelet-bound GPIb α for thrombins.

Numerous studies have suggested previously the presence of a binding site for thrombins in GPIb α . See, for example, Okumura, T. and Jamieson, G.A., Platelet Glycocalicin: A Single Receptor for Platelet Aggregation Induced by Thrombin or Ristocetin, Thromb. Res., 8, 701-706 (1976); Okumura, T. et al., Platelet Glycocalicin: Interaction With Thrombin and Role as Thrombin Receptor of the Surface, J. Biol. Chem., 253, 3435-3443 (1978) referring to receptor activity for thrombin located in the amino terminal 45 kDa fragment of GPIb α ; and also Yamamoto, N. et al., Monoclonal Antibody to Glycoprotein Ib Inhibits Both Thrombin- and Ristocetin- Induced Platelet Aggregations, Thromb. Res., 39, 751-759 (1985).

As described below, the present development provides identification of the high affinity thrombin binding site as consisting approximately of residues 271 to 285 of the GPIb α polypeptide.

An additional aspect of the development provides confirmation that the binding of α -thrombin to platelets can be described by a model based on the existence of three classes of binding sites, those of high affinity (on GPIb α with a dissociation constant, K_d , of about 10^{-10} M), those of medium affinity (K_d of about 10^{-8} M), and of low affinity (K_d of about 10^{-7} M) (see Harmon, J.T. and Jamieson, G.A., J. Biol. Chem., 261, 15928-15933 (1986) for earlier studies).

A further aspect of the development demonstrates that the high affinity binding sites of a platelet for thrombins, located on GPIb α , perform an important role in a specific

platelet activation pathway. Selective inhibition of the high affinity binding site impairs platelet activation. Such impairment can be demonstrated by transient rises in intracellular levels of calcium ions, impairment of dense granule release, and reduction of the amount of fibrinogen binding to platelets and the resultant level of platelet aggregation. However, at sufficient concentration, thrombin can induce platelet activation and aggregation after binding to medium and low affinity receptors.

10 In this regard, it is noted that inhibiting the interaction of GPIIb/IIIa and thrombins at, for example, thrombin concentrations of less than about 1nM, does not prevent binding by thrombins to the medium and low affinity receptors (it is believed that Vu, T-K. E. et al., Cell, 64,1057-1068
15 (1991) have now characterized the independent, medium affinity platelet receptor protein for thrombin) which trigger, independently, activation of platelets at higher thrombin concentrations.

Without being limited as to theory, it is believed that
20 the polypeptides of the invention are effective as antithrombotics in that they inhibit binding of thrombins to GPIIb/IIIa, said binding being important to platelet activation in the early stages of a hemostatic response to a wound or to a diseased vessel. Accordingly, one of the effects of the
25 polypeptides of the invention may be to inhibit the docking of thrombins at the high affinity sites on the platelet surface so that thrombins cannot be oriented to interact with additional components of the hemostatic mechanism. Such regulation is not expected to interfere with participation of
30 thrombin in the clotting cascade when fully activated (such as by a major wound) or at sites in the body removed from where a therapeutic composition is targeted (such as to a site of surgery).

Synthetic polypeptides, in underivatized form, patterned
35 upon all or part of the residue 237 to 291 sequence of GPIIb/IIIa were tested for activity as inhibitors of the interaction between platelet GPIIb/IIIa and thrombins. This region of GPIIb/IIIa was selected for testing because it defines the epitope of LJ-Ib10 monoclonal antibody (described below) which is known

to inhibit thrombin-GPIIb interaction. Preparation of synthetic peptides and methods to test their ability to inhibit binding of thrombins to GPIIb are provided in Example 1. As shown in Figure 1, synthetic peptides containing (or overlapping with) residue sequence Leu²⁷⁵ to Glu²⁸⁵ had inhibitory activity. Representative operable polypeptides, all of which contain Pro²⁸⁰, include Gly²⁷¹ to Glu²⁸⁵ (SEQ ID NO: 3), Pro²⁶⁵ to Glu²⁸⁵ (SEQ ID NO: 2), and Asp²⁶⁹ to Glu²⁸² (SEQ ID NO: 10). Pro²⁶⁵ to Tyr²⁷⁹ (SEQ ID NO: 5) is ineffective as an inhibitor polypeptide. Comparative results are shown in Figure 2 (see also Example 1) for the polypeptide Val²³⁶ to Lys²⁵³ and the aforementioned Pro²⁶⁵ to Glu²⁸⁵ polypeptide.

Additional evidence derived from the practice of the invention suggests that the thrombin-binding region of GPIIb is within residues 265 to 285 thereof and in particular, within residues 275 to 285, and/or polypeptides overlapping therewith, wherein are contained the 3 tyrosine residues 276, 278 and 279 (Figure 1). It has been determined that one or more of these tyrosines are sulfated on the phenolic oxygen of the tyrosine ring. Posttranslational modification of tyrosine by sulfation has been observed also at the binding sites for thrombin of other thrombin-interactive proteins. Accordingly, the invention includes also within its scope sulfated forms (at tyrosine) of aforementioned GPIIb polypeptides.

In connection with providing antithrombotic polypeptides derived from GPIIb containing negatively charged sulfate group on tyrosines, it is noted that other negatively charged functional groups such as, but not limited to, phosphate or carboxyl are expected to be equivalent.

It is noted also that the 45 kDa amino terminal fragment of GPIIb, containing a Leu²⁷⁵ to Glu²⁸⁵ sequence therein, is also effective as an inhibitor of thrombin-GPIIb interaction.

Bifunctional Molecules of the Invention

As mentioned above, a further important aspect of the invention is the recognition that the binding domain of GPIIb for vWF and of GPIIb for thrombins are in close proximity on the GPIIb polypeptide. This high affinity binding site for thrombins is defined, at least in part, by the Leu²⁷⁵ to Glu²⁸⁵

fragment of GPIIb/IIIa and wherein, as expressed in human cells, one or more of Tyr²⁷⁶, Tyr²⁷⁸ and Tyr²⁷⁹ are likely sulfated.

The binding site for vWF on GPIIb/IIIa is located on the amino terminal His¹ to Arg²⁹³ (45 kDa) fragment of GPIIb/IIIa.

- 5 Vicente, V. et al., J. Biol. Chem., 265(1), 274-280 (1990),
Murata, M. et al., J. Biol. Chem., 266, 15474-15480 (1991).

However, the vWF and thrombin binding sites are distinct as evidenced by the behavior of LJ-Ib1 and LJ-Ib10 monoclonal antibodies (Handa, M. et al., J. Biol. Chem., 261, 12579-
10 12585 (1986). Briefly, LJ-Ib1 completely inhibits vWF-GPIIb/IIIa interaction but has no or minimal effect on the binding of thrombins to platelets whereas LJ-Ib10, which interferes with GPIIb/IIIa-thrombin interaction, has no effect on vWF-GPIIb/IIIa interaction. Additionally, the residue 441 to 733 fragment
15 of vWF and α -thrombin can bind simultaneously to platelet GPIIb/IIIa without excluding each other. Accordingly, antithrombotic bifunctional molecules can be constructed comprising two linked domains, a first domain capable of inhibiting the binding of thrombins to platelet glycoprotein
20 Ib/IIIa and a second domain capable of inhibiting the binding of vWF to glycoprotein Ib/IIIa. There follows hereafter information concerning the design of domains of the bifunctional molecules of the invention.

The importance of the present development is based upon
25 the recognition that platelets can become activated (and then aggregated) by more than one pathway or type of stimulus. Providing a therapeutic composition which has the capability of inhibiting more than one such pathway is of substantial clinical importance. The bifunctional molecules of the
30 invention provide such a therapeutic advantage by inhibiting thrombin-induced and vWF-induced platelet activation, two of the most important of the activation pathways. Substantial therapeutic advantage can be attained if both of the above-mentioned activation pathways (through thrombins and vWF)
35 were to be inhibited simultaneously. Such advantage is expected to be greater than that provided by the use of separate or dissociable therapeutic molecules (not covalently bonded to each other). Clinical administration of bifunctional antithrombotic molecules in which the domains

thereof are covalently linked is preferred because it maximizes the antithrombotic effect that can be delivered at a target site such as a lesion, thrombus, or as a target site of surgery.

5 Domains That Target VWF-GPIIb/IIIa Interaction

Interruption of vWF binding to GPIIb/IIIa is predicated on the identification of domains that interact with (bind to) the GPIIb/IIIa polypeptide and prevent vWF from gaining access to or remaining on the receptor site thereof. Representative
10 domains are the following. Polypeptides, whether derivatized or underivatized, that comprise approximately the Arg⁴⁴¹ to Val⁷³³ fragment of the vWF subunit or one or more subfragments thereof containing all or part of the GPIIb/IIIa-binding site thereof.

15 Identification of particular subfragments of vWF subunit that contain all or part of a binding site of vWF for GPIIb/IIIa, and that are suitable as domains in this embodiment of the invention, may be determined by one or more assays well known in the art, including the capability of a domain to inhibit
20 the binding of LJ-Ib1 monoclonal antibody to platelet GPIIb/IIIa, or to inhibit ristocetin or botrocetin-induced binding of ¹²⁵I-labelled multimeric vWF to formalin-fixed platelets. In this regard, preferred species of subfragment of vWF subunit are provided in Example 6. Additional domains effective in
25 the practice of the invention include mutant vWF polypeptide sequence having enhanced affinity, relative to wild type for GPIIb/IIIa, and "derivatives" of vWF polypeptide sequence, as that term has been specially defined herein.

Additionally preferred in the practice of the invention
30 as a domain is an antibody, or an active fragment thereof, having as its epitope, or as part thereof, all or part of the vWF binding site of glycoprotein IIb/IIIa. Use of a complete antibody molecule is consistent with the practice of the invention as long as the patient is monitored for adverse
35 clinical effects resulting from an immune response thereto including those effects mediated by the Fc region of the antibody. Preferred species (see Example 6 for methods of constructing same).

Example 8 describes how active fragments of the LJ-Ib10

antibody (reactive to the binding site of GPIIb/IIIa for thrombins) suitable as domains in the practice of the invention may be prepared. Factors that are important in the design of such active fragments for use in the practice of the invention include

- 1) avoiding use of the F₂ region of the antibody to avoid triggering events such as cell-mediated immune response, or complement reaction;
- 2) small polypeptide fragments of the complete antibody are preferred for the purpose of avoiding production of antibody against the therapeutic fragment by the patient's immune system. In this regard, F₁ fragments (Example 8) or single polypeptide or peptide fragments thereof containing all or part of one or more CDR regions thereof provide effective inhibition peptides.

With respect to the domain of the bifunctional molecules capable of inhibiting binding of thrombins thereto, it is noted that such a domain may function in one of two ways:

- 20 Antibodies, or active fragments thereof that bind to the thrombin binding site of GPIIb/IIIa prevent thrombins from occupying the GPIIb/IIIa site and activating the underlying platelet. Factors useful in the selection of an antibody or an active fragment thereof directed to the vWF-binding site of GPIIb/IIIa are equally applicable to the selection of antibodies or active fragments thereof directed to the thrombin-binding site (such as of LJ-Ib10).

An additional type of molecule suitable as a domain to inhibit binding of thrombins to GPIIb/IIIa is represented by the amino terminal His¹ to Arg²⁹³ fragment of GPIIb/IIIa or a fragment thereof containing all or part of the thrombin binding site thereof. Without limitation as to theory, it is believed that anti-thrombin domains of this type direct thrombins to the domain of the bifunctional molecule patterned on GPIIb/IIIa instead of the underlying GPIIb/IIIa polypeptide receptor of the platelet, thereby avoiding platelet activation.

Mutant and derivatized peptides as those terms have been previously described are also useful in the design of the anti-thrombin domain. Screening of mutant GPIIb/IIIa-derived or

antibody-derived polypeptides for enhanced activity to inhibit binding of thrombins to GPIIb/IIIa may be accomplished using the below described mutagenesis strategy and the assay procedure of Example 1.

5 It is noted that the order of the domains with respect to each other is not important in the design of the bifunctional molecule as long as the linkage thereof is of such design and length so as to provide sufficient flexibility for both domains of the molecule to function.

10 In addition, although the molecules in the above embodiments of the invention are described as "bifunctional," multifunctional or polyfunctional molecules having three or more binding functions are within the practice of the invention and have utility therein. The domains utilized in
15 the practice of the invention have also such additional functions.

The domains of the bifunctional molecules must be linked. Most preferably, the linkage is covalent and may be accomplished according to either of two basic procedures,
20 each of which is readily accomplished and well known in the art.

(A) Encoding DNA for one domain can be combined with encoding DNA for the other domain in a continuous single DNA construct using well known recombinant
25 techniques leading to the expression of a single polypeptide having two domains (see Example 8 for representative methodology). If this method is adopted, it is highly preferred that the encoding DNA encode also a linker peptide sequence between
30 the two polypeptide domains. Such a sequence facilitates each domain adopting a native tertiary conformation, or having sufficient freedom to find and bind to its target site without steric interference from the other domain. Linkers
35 comprising between approximately 10 and 50 amino acids are most preferred but can be optimized for each individual case with appropriate experimentation.

(B) Alternately, the two domains can be expressed or

otherwise provided separately and then chemically linked by attaching between them a spacer or linker, again preferably of suitable length to avoid steric interference between the domains. The two domains can be covalently linked with a linker using attachment at suitable representative reactive groups. Such reactive groups may preexist in the original structure of the polypeptide domains or be covalently added after the isolation or production of the polypeptides.

A representative linker suitable for use either according to method (A) or method (B) comprises a series of glycine residues allowing a high degree of conformational flexibility between the two domains. The necessary length of the linker may be determined by appropriate experimentation.

Additional Aspects of the Development

With respect to the bifunctional molecules and of the polypeptides of the invention, there can be provided for each, including both domains of the bifunctional molecule, mutants of the amino acid sequences which confer thereon enhanced binding affinity for the target receptor(s). There are described hereafter a method for randomly producing mutant vWF-encoding DNA sequences and for screening the resultant expressed polypeptides for those which have enhanced binding affinity for GPIIb α . Similar approaches, based on cloning methodology well known in the art, can be designed to express and then screen mutants of GPIIb α polypeptide for enhanced affinity as domains in the practice of the invention.

30 Mutant Polypeptide Domains Having Enhanced Binding Affinity

The following method is representative of techniques that can be employed to create mutant vWF-derived polypeptide sequences with enhanced GPIIb α -binding affinity.

35 Using vWF DNA from plasmid p5E (which encodes the amino acid sequence comprising mature subunit residues 441 to 733 in which the cysteine residues at positions 459, 462, 464, 471 and 474 thereof are replaced by glycine residues) and random mutant oligonucleotides which can sequentially span

the entire polypeptide, novel variant DNA sequences can be constructed which encode variant vWF-derived polypeptides. The resultant potential therapeutic polypeptides can be screened for relative binding affinity: (1) in direct binding assays for affinity to GPIIb/IIIa; (2) in botrocetin- or ristocetin-induced binding assays; or (3) to conformation dependent vWF-specific antibodies. Random mutagenesis experiments can also be performed using vWF DNA constructs suitable for expression in mammalian cells such as those of Example 7.

Mutant oligonucleotides suitable for site directed mutagenesis protocols and spanning sequential 10 amino acid subdomains of the loop (for example, corresponding to amino acids 690 - 699, 700 - 709, 710 - 719) can be generated using a procedure designed to yield a randomly mutagenized oligonucleotide population. Hutchison, C.A. et al., Proc. Natl. Acad. Sci., USA, 83, 710-714 (1986). The randomized vWF oligonucleotide is then hybridized, for example, to M13mp18 to copy the mutation into a residue 441-733 encoding DNA sequence.

The method of Hutchison, C.A. et al. relies on automated synthesis of the oligonucleotide from the 3' end. In the Hutchison procedure, a random oligonucleotide population suitable for causing permutation of the residues between positions 690 and 710 of the mature vWF subunit can be constructed as follows. The oligonucleotide corresponds to transcribed strand DNA. As the chain is then built stepwise by the nonenzymatic 3'→5' addition of subsequent bases (comprising the part of the vWF loop region to be surveyed), each of the four nucleoside phosphoramidite reservoirs (A,T,G,C) for oligonucleotide synthesis is "doped" with a small amount of each of the other three bases. Incorporation of one of the "doping" nucleotides results in a mutant oligonucleotide. The amount of doping can be adjusted to control results. The resultant randomized population of mutant oligonucleotides is then used in the standard site directed mutagenesis protocol (Example 3) to construct a pool of mutagenized vWF "loop" DNA sequences in M13mp18 corresponding to the mature vWF subunit residue 441-733

fragment and suitable for subcloning into a bacterial expression system.

It is possible to control the number of mutations per molecule by controlling the composition of the base mixtures.

5 For example, it is possible to select for only single base pair substitutions or to select for molecules which have 2, 3, 4, or more mutations. The procedure developed by Hutchison, supra, typically employed solutions of each of the four bases in which approximately 1.5% impurity of each of
10 the other three bases contaminates the original base solutions. Mutagenesis using this particular doped mixture resulted in roughly 41% of clones with no base substitutions, 40% with one, 15% with two, 3% with three and 0.7% with four (for target nucleotide sequences corresponding to 10 amino
15 acids).

The resultant mutant M13mp18 populations are then subject to restriction, and the mutagenized DNA sequences are inserted into vectors or plasmids such as pET-3A for expression in host bacterial cells. Large scale screening of
20 mammalian clones is generally much more difficult than for bacterial clones. However, promising mutations identified in bacterial constructs may later be inserted into mammalian or other eucaryotic host cells for further testing or for commercial-scale polypeptide production.

25 The mutant clones can then be screened in GPIIb α binding assays or in binding assays with vWF-specific monoclonal antibodies. Mutant clones having cell lysates which exhibit enhanced platelet binding or antibody response can be sequenced to determine the amino acid alteration(s)
30 responsible for the mutant phenotype. In this way, a very systematic analysis of the loop region of vWF can be performed and mutations which alter the binding of vWF to GPIIb α can be identified.

The mutagenesis technique is particularly applicable to
35 permuting the amino acid sequence regions of the mature subunit believed to contain actual GPIIb α binding (interaction) sites for the purpose of enhancing their GPIIb α affinity.

Screening of Mutant vWF-Derived
Polypeptides for Enhanced GPIIb Binding Activity

There is hereafter described a method for screening randomly mutagenized mature vWF subunit polypeptide sequences for enhanced GPIIb-binding activity and resultant enhanced utility as domains of the invention.

To perform the assays, a device used for the enzyme-linked immunofiltration assay technique (ELIFA), Pierce Chemical Co., Rockford, IL, can be adapted in combination with immobilization of the mutant vWF-derived polypeptides to be tested. It is considered most efficient to test initially the effect of mutant codons on vWF polypeptides expressed from bacterial constructs and to then copy potentially useful mutations (using, for example, mutagenesis in M13mp18 vehicle) into a mammalian expression construct. High levels of mutant vWF polypeptides corresponding to mutant DNA sequences can be expressed from pET-3A type bacterial expression plasmids such as p5E. Mutant polypeptides constitute a major portion of host E.coli cell lysates and can be screened readily for GPIIb affinity.

Accordingly, site directed mutagenesis can be performed following the procedure of Example 3, using as template in M13mp18 the vWF fragment corresponding to p5E expression plasmid which because of the use of BamHI linkers in assembly of p5E is recovered therefrom and inserted into M13mp18 as an XbaI/HindIII fragment. For the oligonucleotide pool, oligonucleotides each having randomly mutagenized residue 690 to 710 sequences are used.

The mutagenized population of M13mp18 constructs can be cloned into pET-3A plasmids after which the expression plasmids can be transformed into E.coli BL21 (DE3). Preparation of mutant polypeptide extracts from E.coli BL21(DE3) for screening follows the procedure of Example 3, with the final step being solubilization of extracted inclusion body material with 8 M urea at room temperature for 2 hours.

Resultant extracts of expressed mutant p5E-type vWF polypeptides are immobilized following the manufacturer's instructions onto a nitrocellulose membrane (0.45 μ pore size)

using 96-well sample application plates (Easy-Titer® ELIFA System, Pierce Co., Rockford, IL) and a vacuum chamber. Commercially available pump materials can be used. The apparatus is suitable for screening large series of clone
5 lysates in an ELIFA or dot blot system and allows also quantitative transfer of sample fluids to underlying microtiter wells without cross contamination.

Immobilization of the vWF polypeptides is accomplished by causing a suitable volume, such as 200 μ l, of each
10 resuspended inclusion body pellet material (in 8 M urea) to be vacuum-drawn through the individual wells to the nitrocellulose membrane over a 5-minute period. Several 200 μ l volumes of Hepes-buffered saline are then drawn through the membrane to remove urea.

15 The protein binding capacity of the membrane is then saturated by passing through it three consecutive 200 μ l aliquots of HEPES/BSA buffer having a pH of 7.4 and comprising 20 mM Hepes, 150 mM NaCl, and 1% w/v bovine serum albumin (Calbiochem, La Jolla, CA).

20 After completion of the above procedure to minimize background caused by nonspecific interaction, a 50 μ l volume of HEPES/BSA containing botrocetin (at approximately 0.5 μ g/ml) or containing ristocetin (at approximately 1 mg/ml) can be vacuum drawn through the nitrocellulose membrane again
25 over a 5-minute period. The ristocetin-induced precipitation of bacterially-expressed vWF polypeptides observed under some test conditions is not expected to cause difficulty in this assay as the polypeptide is already immobilized.

GPIb(α) represented by its external domain,
30 glycalicin, or the 45 kDa tryptic fragment thereof is next applied to the nitrocellulose using the vacuum system and the 96-well-plate. The GPIb α fragments are purified and ¹²⁵Iodinated by standard procedures (Vicente, V. et al., J. Biol. Chem., 265, 274-280 (1990)). Fifty μ l aliquots of
35 HEPES/BSA containing ¹²⁵I-GPIb α fragments (0.25 μ g/ml having a recommended specific activity of between approximately 5×10^8 and approximately 5×10^9 cpm/mg) can then be vacuum drawn through the nitrocellulose filter over 5 minutes.

The membrane is then allowed to dry and discs

corresponding to the position of each application well are cut out and counted in a γ scintillation spectrometer to determine bound radioactivity. An autoradiograph of the membrane can also be obtained before cutting out the discs in order to ascertain that there was no leakage of radioactivity from one well to another. The counting process may be facilitated by scanning the developed autoradiogram in a densitometer to digitize the intensity of developed spots. As long as the autoradiogram is not excessively overdeveloped, beyond the linear region of response, useful qualitative results are obtained.

An alternate procedure to derive from individual host E.coli clones an impure extract which can be screened in immunoblot or dotblot procedures is as follows. A large set of individual E.coli colonies carrying separate randomly mutagenized vWF inserts is picked and grown overnight as separate cultures. The cultures are then diluted 1:100 and grown to an OD₆₀₀ of 1.0. vWF fragment synthesis is induced by adding isopropyl- β -d-thiogalactopyranoside (IPTG), U.S. Biochemicals, Cleveland, OH, to 5 mM and continuing growth for approximately 2.5 hours. The cells are harvested by centrifugation for 1 minute at 10,000 g and then washed and repelleted (at 10,000 g) 3 times with phosphate buffered saline (0.14 M NaCl, 0.1 M Na₂HPO₄, pH 7.0). The bacterial pellet is then solubilized by boiling for 10 minutes in a buffer comprising 0.01 M NaH₂PO₄, 10 mM Na₂EDTA, 1% (w/v) sodium dodecylsulfate, pH 7.0. The incubation is continued for 2 hours at 60°C in the presence also of 10 mM dithiothreitol (DTT). Suitable volumes (such as 200 μ l) of such extracts can be used directly in ELISA apparatus or dot immunoblot analyses. Prior to adding ¹²⁵I-GPIb α to the plate, several rinses of Hepes-buffered saline are washed through the wells.

vWF derived polypeptides from colonies representing the most intense response are selected for confirmation of enhanced binding using methods such as subjecting purified or partially purified extracts therefrom as appropriate to: (A) immunoblotting with an appropriate antibody; (B) assaying for ability to inhibit botrocetin-induced vWF binding to

formalin-fixed platelets on a dose dependent basis; or (C) assayed for ability to inhibit the binding of anti GPIIb/IIIa monoclonal antibodies to platelets.

Clones which confer enhanced positive responses in these systems are then subjected to standard DNA sequencing procedures to identify the vWF gene mutations responsible for the mutant properties. The appropriate mutations may be copied into a vWF DNA sequence within a plasmid (such as a pAD5/WT-pCDM8^{neo} expression plasmid) suitable for expression in CHO-K1 cells (see Azuma, H. et al., J. Biol. Chem., 266(19), 12342-12347 (1991)). Further characterization, such as enhanced potential for induction of platelet aggregation by 116 kDa homodimers thereof can then be performed.

15 Organic (Peptidomimetic) Analogs of the Bifunctional Molecules and Polypeptides of the Invention

Interest in the provision of organic analogs of therapeutic polypeptides can be traced to the recognition that morphine achieves its analgesic action by mimicing the structure of certain natural peptide analgesics of brain tissue, the endorphins. It is recognized that such organic analogs possess several advantages over their polypeptide counterparts including (1) a longer half life before metabolism, or nonspecific binding make the natural molecule unavailable; and (2) a lesser likelihood of inducing an immune response in the patient that would limit the utility thereof. General principals are now available to guide the synthesis of such organic analogs. See, for example, Farmer, P.S., Gridging the Gap Between Bioactive Peptides and Drug Design, vol. X, 119-143, Academic Press (1980); Rudinger, J., The Design of Peptide Hormone Analogs in

_____, chapter 9, 319-4____, (19____); SYBYL version 5.32, Molecular Modeling Software, _____

_____, Associates, Inc., St. Louis, MO 63144, copyright 1991; Abola, E.E. et al., "Protein Data Bank" in Crystallographic Databases - Information Content, Software Systems, Scientific Applications, Allen, F.H. ed., Data Commission of the International Union of Crystallography, Bonn, pp. 107-132 (1987).

Manipulation of Cysteine Residues of Bifunctional Molecules or of Polypeptides of the Invention

With respect to the provision of polypeptides of the invention or domains of bifunctional molecules of the invention in which certain cysteine residues thereof have been inactivated (my missense mutation with site-directed mutagenesis) so as to avoid undesired crosslinking, the following should be noted. There are available also numerous other well known procedures that can be used to inactivate cysteine residues. One such technique involves treatment of cysteine residues with a reducing agent such as, for example, 6-mercaptoethanol or dithiothreitol "DTT" followed by permanent alkylation (for example, with iodoacetamide). Numerous other covalent labels may be attached to the target cysteine residues, the only requirements being that the label be applied under pH conditions which do not irreversibly denature the target protein. The attachment is of a kind which, under the conditions to which the fragment is exposed during further processing or storage, will not allow chemical reaction with other cysteine residues. Such covalent labelling procedures are generally known in the art and include also, for example, reaction with (A) iodoacetic acid or (B) iodinating agents such as iodo fluorescein. Alteration can be accomplished also by site directed mutagenesis of an encoding DNA or by deleting sequence positions corresponding to cysteine.

Additional Strategies for Antithrombotic or Cancer Therapy

The use of the bifunctional molecules and of the polypeptides of the invention has been described in terms of achieving, as an example, a beneficial antithrombotic response in the presence of low concentrations of thrombin without preventing platelet response to higher concentrations of thrombin. Accordingly, administration of a GPIIb/IIIa receptor-selective composition to a patient (for example, undergoing surgery or afflicted with thrombosis) would not only provide desired localized or immediate antithrombotic effects, but could minimize the risk of systemic antihemostatic effects that would prevent response to higher concentrations of thrombin associated with a major bleeding

event that would fully activate the clotting cascade, or response at sites in the body far removed from the initial target site.

Use of the bifunctional molecules and polypeptides of the invention is not limited, in approach, solely to inhibition of the binding of thrombins or vWF at GPIIb sites on platelets. Expression of GPIIb is not limited to platelets or to the megakaryocytic cells from which platelets are produced. It has been determined that GPIIb is also expressed in endothelial cells - Konkle, B.A. et al., J. Biol. Chem., 265(32), 19833-19838 (1990) have identified mRNA for GPIIb in human umbilical vein endothelial cells and in vascular endothelium of tonsil. GPIIb was also identified by the authors immunochemically in blood vessels of human tonsil.

It is expected that GPIIb, or molecules with homologous structure and function, are also present on other normal and pathological (including cancerous) cells. Asch, A.S. et al., J. Clin. Invest., 81, 1600-1607 (1988) have reported identification of a GPIIb-like protein in human smooth muscle cells. Grossi, I.M. et al., FASEB J., 2, 2385-2395 (1988) have reported that certain carcinoma cell lines express proteins which react with anti-GPIIb monoclonal antibodies. These antibodies were also reported to inhibit tumor-associated platelet aggregation.

In view of the role that α -thrombin plays in cell activation (see Vu, T.H. et al., Cell, 64, 1057-1068 (1991)), and since GPIIb or molecules with homologous structure and function contribute to production of thrombin interaction with cells, the bifunctional molecules or polypeptides of the invention have utility in modulating the response to thrombins and/or vWF of cells in blood vessel walls, such as endothelial cells or smooth muscle cells. This is expected to have clinically useful consequences, both for the short term and long term response of vessels to thrombogenic and atherogenic stimuli. For example, endothelial cells stimulated by cytokines at a site of inflammation or as a consequence of an immune-mediated insult may express GPIIb (or molecules with homologous structure and function). The

binding of vWF and/or thrombins to the expressed receptor may lead to further activation of the endothelial cells and/or mediate the attachment of platelets to these cells.

5 Platelets become activated as a consequence of adhesion and, thus release growth factors and other factors that may initiate the proliferation of smooth muscle cells in the vessel wall. Smooth muscle cells may also be further activated as a consequence of thrombin binding to expressed GPIb α , or molecules with homologous structure and function.

10 These processes may play an important role in the response to atherogenic stimuli and, therefore, may have a long-term effect on the development and progression of vascular diseases leading, eventually, to thrombosis. Moreover, thrombin bound to endothelial cells represents a potent

15 catalyst for a series of biochemical enzymatic reactions important for the formation of fibrin. fibrin deposition on the vessel wall may have a pathogenetic role both in the long term development of atherosclerotic lesions as well as in acute occlusive events. The latter may take place in vessels

20 stenosed by advanced atherosclerotic processes, usually as a consequence of rupture of an atherosclerotic plaque but, possibly, also following adhesion of platelets to damaged and stimulated endothelial cells. It is evident from the above discussion that the inhibition of thrombin and/or vWF binding

25 to endothelial cells and other cells of the vessel wall may influence the onset, progress and terminal occlusive events of atherosclerotic diseases. It should also be mentioned that the inhibition of thrombin binding to GPIb α or homologous molecules may have an indirect beneficial

30 antithrombotic effect by favoring the binding of thrombins to thrombomodulin. After the latter interaction takes place, thrombin itself acquires an antithrombotic activity through the activation of the Protein C pathway of inhibition of coagulation cofactors.

35 In addition, inhibition of the binding of thrombins or vWF to GPIb α receptors on tumor cells can inhibit certain behaviors of tumor cells, thereby influencing their metastatic potential. For example, it has been noted that platelets interact with tumor cells and influence their

ability to penetrate the vessel wall and colonize tissues, the central event in the establishment of a metastatic area of tumor growth. Moreover, the assembly of fibrin around a tumor metastasis may be one of the mechanisms by which tumor cells protect themselves from defense mechanisms of the host organism after seeding new tissues. Consequently, the inhibition of the binding of thrombins and vWF to GPIIb/IIIa or homologous molecules on tumor cells may have clinical advantages in the treatment of cancer patients by limiting the ability of primary tumors to grow and metastasize.

Additional Antibodies with Therapeutic Activity

Antibodies, and particularly conformation dependent antibodies, are powerful tools for analyzing the structure and function of macromolecules. By blocking macromolecular interactions, antibodies can also have important therapeutic and diagnostic utility.

Accordingly, this invention includes within its scope antibodies that are specific for all or part of any of the bifunctional molecules or polypeptides of the invention, including, therefore, antibodies that recognize idiotypes, all said antibodies being made by a process which involves immunizing animals with a bifunctional molecule, or polypeptide, of the invention or a component thereof.

Therapeutic compositions

One or more of the bifunctional molecules or polypeptides of the present invention can be formulated into pharmaceutical preparations for therapeutic, diagnostic, or other uses. To prepare them for intravenous administration, the compositions are dissolved in water typically containing also physiologically compatible substances such as sodium chloride, glycine, and the like resulting in a solution having a pH, ionic strength, and osmotic potential compatible with therapeutic use (such concentrations being well known in the art), said water and any other physiologically compatible substances comprising a pharmaceutically acceptable carrier.

With respect to the therapeutic use of the bifunctional molecules or polypeptides of the invention, the amount to administer for the prevention or inhibition of thrombosis will depend upon the affinity of the molecule for platelet

GPIIb in vivo, and/or for other macromolecules that participate in hemostasis and thrombosis in the body, and on the severity with which the patient is subject to thrombosis. Said amount can be determined readily for any particular patient.

It is also within the practice of the invention to provide a therapeutic composition containing one or more of the bifunctional molecules or polypeptides of the invention and also additional therapeutic substances. Such additional substances include heparin and other anticoagulants, aspirin or other antiplatelet drugs, or tissue plasminogen activator or other prefibrinolytic drugs.

The following Examples are representative of the practice of the invention.

Example 1 - Inhibition of α -thrombin binding to platelets by synthetic peptides

This example demonstrates the effect of two synthetic peptides (patterned upon the amino acid sequence of GPIIb) on the binding of α -thrombin to platelets. The peptides were synthesized chemically following the method of Houghten, R.A., Proc. Natl. Acad. Sci. USA, 82, 5131-5135 (1985) with further purification as described in Vicente, V. et al., J. Biol. Chem., 265(1), 274-280 (1990). Accordingly the resultant peptides lack any derivatizing groups such as sulfate or glycosyl moieties which may be added to polypeptides in mammalian cells as a result of posttranslational modification thereof. The GPIIb peptides synthesized were Pro²⁶⁵-Glu²⁸⁵ (SEQ ID NO: 2) and Val²³⁶-Lys²⁵³ (SEQ ID NO: 4).

α -thrombin was prepared according to the method of Fenton, J.W. et al., J. Biol. Chem., 252, 3587-3598 (1977). Iodination of α -thrombin was accomplished according to the method of Fraker, P.J. and Speck, J.C., Biochem. Biophys. Res. Commun., 80, 849-857 (1974). Washed platelets were prepared from normal human donors according to the method of Walsh, P.N. et al., Br. J. Haematol., 36, 281-298 (1977) with modifications as described in Ruggeri, Z.M. et al., J. Clin. Invest., 72, 1-12 (1983).

To perform the assay the following procedure was used.

Washed platelets were suspended at a concentration of 1.875×10^{12} /liter in binding buffer, hereinafter (136 mM NaAcetate, 25 mM Tris base, 0.6% (w/v) polyethyleneglycol 6,000, pH 7.3) and incubated at 37°C for 10 minutes immediately prior to

5 use. There were also prepared the following stock solutions:

- (A) bovine serum albumin, "BSA" (Fraction V, Calbiochem, San Diego, CA) at 4.1% (w/v) in binding buffer;
- 10 (B) peptides - stock solutions thereof were prepared in "Hepes buffer" consisting of 20 mM Hepes, 150 mM NaCl, pH 7.3;
- (C) ^{125}I - α -thrombin-stock solution of about 0.1 mg/ml ($2.7 \mu\text{M}$) in 20 mM Tris base, 0.75 M NaCl, 8% (w/v) BSA, pH 7.3;
- 15 (D) prior to assay, ^{125}I - α -thrombin is diluted from (C) above to a secondary stock solution (binding buffer/4.1% BSA) in which its concentration is 5.75 nM.

The assay is then performed by adding, in rapid
20 succession, 50 μl binding buffer; 20 μl of 4.1% BSA in binding buffer; 20 μl washed platelet suspension (final assay count, 3×10^{11} /liter); 25 μl of peptide stock (after dilution resulting in peptide assay concentrations of 50, 100, 200, 400, etc. μM) in "Hepes buffer" or 25 μl Hepes buffer as the
25 control; and finally 10 μl ^{125}I - α -thrombin stock solution (D) resulting in a final α -thrombin concentration of 0.46 nM.

At the end of the incubation, platelet-bound and free α -thrombin were separated by centrifugation of the platelets (for 4 minutes at 12,000 g through a 20% sucrose gradient in
30 Hepes buffer consisting of 20 mM Hepes, 150 mM NaCl, pH 7.3). The radioactivity associated with the platelet pellet was counted in a γ scintillation spectrometer, and bound α -thrombin in the mixtures containing peptide was expressed as percentage of that bound to platelets in the control mixture
35 (without peptide, as shown on the ordinate of Figure 2).

The average inhibitory effect of Pro²⁶⁵-Glu²⁸⁵ corresponds to approximately 64% inhibition of the total amount of α -thrombin otherwise bound to the platelets, said incomplete inhibition being consistent with the fact that platelets

possess more than one class of thrombin binding sites. A concentration of 80 μ M of the peptide was needed to achieve approximately 50% of inhibition under the assay conditions used. The Val²³⁶-Lys²⁵³ peptide had no effect as a binding inhibitor under these assay conditions (Figure 2 shows one representative trial for this peptide; the average of four trials being 149% of control). Also without effect at a 400 μ M concentration was peptide Asp²³⁵-Lys²⁶². Peptide Phe²¹⁶-Thr²⁴⁰ achieved a negligible inhibitory effect, approximately 10%, at the 400 μ M concentration. These results are representative of those achieved with repeated trials, there being some variation between each preparation of platelets, such variation being known in the art.

Example 2 - Preparation of a Large Polypeptide Domain of GPIIb α Useful as a Component of Bifunctional Antithrombotic Molecules

Step 1. Construction of a DNA sequence for expression of the mature His¹-Leu⁶¹⁰ polypeptide

Based on the published GPIIb α cDNA sequence of Lopez, J.A. et al., Proc. Natl. Acad. Sci. USA, 84, 5615-5619 (1987), two flanking oligonucleotides were synthesized for the amplification in a polymerase chain reaction of a region of the GPIIb α gene which it was believed would be suitable as a probe to screen a human genomic lambda (λ) phage library.

Accordingly, human genomic DNA was subjected to enzymatic amplification in a polymerase chain reaction according to the method of Saiki et al., Science, 239, 487-491 (1988). The procedure utilizes a double stranded GPIIb α DNA sequence, a subsegment of which is to be amplified, and two single stranded oligonucleotide primers which flank the ends of the subsegment. The primer oligonucleotides (in the presence of a DNA polymerase and deoxyribonucleotide triphosphates) were added in much higher concentrations than the DNA to be amplified. The vast majority of polynucleotides which accumulate after numerous rounds of denaturation, oligonucleotide annealing, and synthesis represent the desired double stranded cDNA subsegment suitable for further propagation by cloning.

PCR reactions were performed with a DNA thermal cycler (Perkin Elmer Co., Norwalk, CT/Cetus Corporation, Berkeley,

CA) using Taq polymerase (Thermus aquaticus). The reactions were run in 100 μ l volumes containing 1.0 μ g of human genomic DNA, 1.0 μ g of each synthetic oligonucleotide primer, and buffer consisting of 50 mM KCl, 10 mM Tris·HCl (pH 8.3), 1.5 mM MgCl₂, 0.1% gelatin (BioRad Co., Richmond, CA) and 200 mM of each dNTP. PCR conditions were 35 cycles of 30 seconds at 94°C, 30 seconds at 52°C and 1 minute at 72°C. Amplified fragments were then purified and isolated by electrophoresis through a 2% agarose gel, Maniatis et al., Molecular Cloning, A Laboratory Manual, 164-170, Cold Spring Harbor Lab., Cold Spring Harbor, NY (1982).

Specifically, the following oligonucleotides were synthesized by the phosphoramidite method, Sinha et al., Tetrahedron Letters, 24, 5843 (1983) using a model 380B automated system, Applied Biosystems, Foster City, CA.

The oligonucleotides selected were:

Oligonucleotide (A) (SEQ ID NO: 11)

| | | | |
|----|--------|-------------------------------------|----------|
| | 644 | | 674 |
| | ↓ | | ↓ |
| 20 | 5' - G | AAT CTG ACA GCG CTG CCT CCA GAC CTG | CCG - 3' |
| | | Asn | Pro |
| | | 21 | 30 |

and

Oligonucleotide (B) (SEQ ID NO: 12)

| | | | |
|----|---------|---------------------|----------|
| 25 | 3' - GC | GAC GGA GAA CCA CGG | GAC - 5' |
| | ↑ | | ↑ |
| | 925 | | 944 |

| | | | |
|----|---------|---------------------|----------|
| 30 | 5' - cg | ctg cct ctt ggt gcc | ctg - 3' |
| | | Leu | Leu |
| | | 115 | 120 |

Oligonucleotide (A) is equivalent to non-transcribed strand DNA (coding strand) for nucleotides 644-674 (using the numbering system of Wenger et al., Biochem. Biophys. Res. Comm., 156(1), 389-395 (1988), for the GPIb α gene).

Oligonucleotide (B) is shown 3' \rightarrow 5' and is equivalent to the transcribed strand (noncoding DNA). The corresponding coding strand is shown 5' \rightarrow 3' in lower case letters. Nucleotide positions are according to Wenger et al.

T₄ kinase was used to add phosphate groups to each end of the amplified fragment. T₄ ligase was used to blunt end

ligate the fragment into the SmaI site within the multiple cloning sequence of the double stranded replicative form of M13mp18 bacteriophage. The ability to isolate a stable single stranded (+) form of the virus is particularly useful to verify the integrity of any cloned sequences therein. See Messing, J. Meth. Enzymology, 101, 20-78 (1983), and Yanish-Perron et al., Gene, 33, 103-109 (1985). Accordingly, the GPIb α DNA insert was completely sequenced using single stranded dideoxy methodology (Sanger et al., Proc. Natl. Acad. Sci. USA, 74, 5463-5467 (1977) utilizing the single stranded (+) form of M13mp18. Sequencing in M13mp18 established that the GPIb α insert was 301 base pairs in length, indicating that the corresponding cDNA region, Lopez et al., did not involve an intron boundary. The 301 base pair (bp) fragment was then subjected to nick translation for incorporation of ^{32}P -labelled nucleotides, thus converting the fragment into a radiolabelled probe, Rigby et al., J. Mol. Biol., 113, 237 (1977).

A human genomic λ phage library (using Lambda FixTM, Stratagene, La Jolla, CA) was prepared using an EcoRI partial digest of human cell DNA. The library was screened following the hybridization and plaque purification procedure of Benton et al., Science, 196, 180-182 (1977) using E. coli strain LE 392 as host. Screening with the 301 bp fragment resulted in the isolation of 6 positive clones after 4 cycles of plaque purification.

In order to conduct the library screening for each positive clone, an appropriate dilution of λ phage was incubated with bacteria at 37°C for 20 minutes with constant shaking. Melted agarose was added to this mixture and the entire contents spread onto a petri dish with a hard agar base. The plates were incubated overnight at 37°C. An imprint of the bacteriophage plaques thus obtained was produced by gently placing a nitrocellulose filter onto the surface of the plate. Phage particles and DNA were transferred to the filter by capillary action in an exact replica of the pattern of plaques. After denaturation with NaOH, the DNA was irreversibly bound to the filter by baking and was then hybridized to the ^{32}P -labelled probe. Unbound

probe was washed away and the filters were exposed to film. Plaques which were positive for hybridization were identified by aligning the film with the original agar plate. These plaques were individually picked and amplified. In general
5 the initial plating density of phage was such that individual plaques could not be picked but instead an area comprising several different phage species was picked. This mixture was amplified and replated at low density to be rescreened to determine which initial positives were true positives and to
10 "plaque" purify each positive. After 3 rounds of such rescreening individual positively hybridizing phage were isolated for further characterization.

Purified λ DNA was then isolated from each positive λ clone by precipitating phage from respective lysed E. coli LE
15 392 samples following the procedure of Maniatis et al., at 76-85.

One μ g samples of DNA from each of the six positive λ clones were then digested with EcoRI. The EcoRI digests were then separated according to molecular weight by electro-
20 phoresis in agarose, followed by transfer to nitrocellulose for detection by autoradiography using the 32 P-labelled 301 bp fragment. Southern, J. Mol. Biol., 98, 503 (1975). An approximate 6000 base pair EcoRI fragment was recognized.

The approximate 6000 base pair fragment visualized and
25 extracted from an agarose gel was then cloned into pBluescript KS- plasmid (Stratagene Co., La Jolla, CA) at its EcoRI site. The plasmid was then propagated in E. coli strain XL-1 Blue (Stratagene Co.). Plasmids were recovered from host E. coli by an alkaline cell lysis procedure,
30 Birnboim and Doly, Nucleic Acids Research, 7, 1513 (1979) followed by purification by CsCl/ethidium bromide equilibrium centrifugation according to Maniatis et al., at 1.42.

Plasmid so isolated was then digested with BamHI and BglII creating a 2161 base pair fragment (nucleotides 503 to
35 2663 using the numbering system of Wenger et al.) which fragment extends from upstream above the initiating MET¹ codon (nucleotides 537-539) to downstream below the LEU⁶¹⁰ codon (nucleotides 2412-2414) and the TGA translation stop codon (2415-2417). The BamHI site of the fragment

corresponds to nucleotides 502-507 and the BglIII site thereof nucleotides 2658-2664.

The 2161 bp fragment was then cloned into the BamHI site of pBluescript KS- (Stratagene Co., La Jolla, CA) as a BamHI-BglIII fragment. Since BamHI and BglIII restriction sites contain identical internal sequences GATC/CTAG, a BglIII restricted site may be annealed into a BamHI site. The fragments were ligated with T₄ DNA ligase, however the integrity of the affected BglIII end was not restored.

10 Hybridization with the 301 base pair probe and sizing on agarose were repeated. The plasmids were propagated in E. coli XL-1 Blue.

Restriction mapping was then performed to select a clone of E. coli XL-1 Blue (Stratagene) in which the GPIb α DNA within a contained pBluescript KS- plasmid possessed an insert orientation such that the XhoI site of the polylinker would be upstream (5') from the insert and the NotI site would be downstream (3') therefrom. The XhoI-NotI fragment was used as follows to create a suitable expression plasmid.

20 Step 2. Construction of plasmids
 for integration into mammalian cells

A selection procedure based on aminoglycosidic antibiotic resistance was designed to select, continuously, for transformants which would retain a suitable GPIb α expression plasmid.

pCDM8 vector, (developed by Seed et al., Nature, 329, 840-842 (1987) and available from Invitrogen, San Diego, CA) was modified by Dr. Timothy O'Toole, Scripps Clinic and Research Foundation, La Jolla, CA to include a neomycin resistance gene (phosphotransferase II) that was cloned into the BamHI restriction site of pCDM8 as a part of a 2000 base pair BamHI fragment. The protein produced by the neomycin (neo) gene also confers resistance against other aminoglycoside antibiotics such as Geneticin[®] G418 sulfate

30 (Gibco/Life Technologies, Inc., Gaithersburg, MD).

Several other suitable expression vectors containing neomycin resistance markers are commercially available. Examples include pcDNA 1^{neo} (Invitrogen, San Diego, CA), Rc/CMV (Invitrogen, San Diego, CA) and pMAM^{neo} (Clontech, Palo

Alto, CA). If necessary the GPIba fragment may be differently restricted or modified for expression capability in these other expression plasmids.

The XhoI-NotI fragment from pBluescript KS- plasmid was inserted into pCDM8^{neo} which had been restricted with XhoI and NotI. Ampicillin sensitive E. coli strain XS-127 cells (Invitrogen, La Jolla, CA) were transformed with the resultant ligated DNA mixture following the method of Hanahan, J. Mol. Biol., 166, 557-580 (1983).

10 Plasmids from resultant colonies were characterized by restriction mapping and DNA sequencing to identify colonies which contained the intended insert. One such plasmid (designated pMW1), was maintained in E. coli strain XS-127, and was selected for mammalian cell transformation
15 procedures.

Prior to use in transforming mammalian cells, supercoiled plasmids (pMW1) were recovered from host E. coli by the alkaline cell lysis procedure of Birnboim and Doly followed by purification by CsCl/ethidium bromide equilibrium
20 centrifugation according to Maniatis et al., at 1.42.

Step 3. Transformation of Chinese hamster ovary cells

pMW1 was introduced into CHO-K1 Chinese hamster ovary cells (ATCC-CCL-61) by a standard calcium phosphate-mediated transfection procedure. Chen et al., Mol. Cell. Biol., 7(8),
25 2745-2752 (1987).

CHO-K1 cells were grown to confluence at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Gibco/Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco), 0.5 mM of
30 each nonessential amino acid (from NEAA supplement, Whittaker, Walkersville, MD) and 2.5 mM L-glutamine under a 5% CO₂ atmosphere, trypsinized as elaborated below, and then subcultured 24 hours prior to transformation at a density of 1.25 x 10⁵ cells per 60 mm tissue culture dish (approximately
35 25% of confluence). CHO-K1 cells have a doubling time in DMEM/10% FCS of approximately 16 hours under these conditions.

To accomplish transformation, pMW1 plasmids were recovered from cultures of E. coli strain XS-127, according

to the method of Birnboim and Doly, Nucleic Acids Research, 7, 1513 (1979) as described above. Ten μ g of plasmids were applied to the cells of each 60 mm dish in a calcium phosphate solution according to the method of Chen et al.

5 After inoculation with plasmid the cells were maintained in DMEM/10% FCS at 37°C in a 5% CO₂ atmosphere.

Approximately 48 hours post-transfection and after growth at 37°C in a 5% CO₂ atmosphere, the cells were trypsinized as follows. Growth medium for each dish was

10 replaced by 3 ml of a solution of phosphate-buffered saline (37 mM NaCl, 27 mM KCl, 4.3 mM Na₂HPO₄·7H₂O/1.4 mM K₂HPO₄, pH 7.4) containing also 0.25% trypsin, 0.2% (w/v) EDTA. Trypsinization was conducted for 3 minutes. The trypsin-containing medium was removed and the dishes were then placed

15 in the incubator for a further 15 minutes after which the cells were resuspended in DMEM containing 10% FCS. The cells from each dish were then split 20 fold, and plated at a density of approximately 1.2×10^4 cells/60 mm dish (approximately 2% of confluence).

20 Production of stable transformants, which have integrated the plasmid DNA, was then accomplished by adding Geneticin® G418 sulfate to the 60 mm dishes to a concentration of 0.8 mg/ml. Growth was continued for 14 days at 37°C in a 5% CO₂ atmosphere. Surviving independent

25 colonies were transferred to 12 well plates using cloning rings and then grown for another seven days in DMEM/10% FCS supplemented with 0.8 mg/ml of Geneticin®. Under these conditions 3 to 7 surviving colonies per plate were apparent after 10-14 days. Approximately 100 stable transformants can

30 be isolated from each original 60 mm dish originally containing approximately 5×10^5 cells at a plate density of approximately 70% of confluence.

Based on screening with the LJ-P3 anti-GPIb α monoclonal antibody, more than 50 percent of G418-resistant cell lines

35 produce antigen corresponding to mature GPIb α polypeptide. The specific geometry of integration of each clone presumably prevents expression in all cases. Stable transformants were then cultured and maintained at all times in medium containing Geneticin® G418 sulfate (.8 mg/ml) to apply

continuous selection.

Colonies expressing the recombinant mature GPIb α polypeptide were detected by dot-blot analysis on nitro-cellulose after lysis in buffer. As a control, recombinant
5 cell extracts were compared with that from nontransfected CHO-K1 cells.

To prepare cell extracts, non-transfected or transfected CHO-K1 cells were harvested with 3.5 mM EDTA and resuspended in 0.25 M Tris-HCl pH 7.5 (10^3 cells/ μ l). Cells were lysed
10 by three cycles of freezing and thawing and centrifuged at 12,000 g to remove cell debris. The resulting supernatant was kept at -70°C as cell extract.

To prepare samples of culture medium containing secreted GPIb α antigen, 80% confluent non-transformed or transformed
15 CHO cells grown in medium containing FCS were washed once with serum-free medium, and then fed with serum-free medium supplemented with L-glutamine and nonessential amino acids. After 24 hours, the medium was collected and centrifuged at 12,000 g to remove cell debris. The corresponding
20 supernatants were pooled and stored at -70°C until used. Monoclonal antibodies LJ-Ib1 Handa et al. and LJ-P19, which recognize GPIb α native conformation were used as primary antibody. The secondary antibody (125 I-rabbit anti-mouse IgG) which had been labelled by the method of Fraker et al.,
25 Biochem. Biophys. Res. Commun., 80, 849-857 (1978) was incubated for 2 hours at 25°C on a nitrocellulose sheet. After rinsing, the nitrocellulose was developed by autoradiography to identify colonies expressing GPIb α antigen.

30 Extracts from pMW1-transformed cells contain as a minor component a glycoprotein Ib α antigen having an approximate apparent molecular weight of 79 kDa as measured by SDS-polyacrylamide gel electrophoresis under reducing or non-reducing conditions. This band represents full length
35 glycoprotein Ib α chain (residues 1-610 and minus the signal peptide) without glycosylation. The 79 kDa polypeptide reacts with anti-GPIb α monoclonal antibody LJ-IB α 1 which has its epitope in the amino terminal region of the denatured (α) polypeptide, whether in reduced or unreduced form. The

relatively small proportion of this species indicates its inherent instability and rapid proteolytic processing. Components of oligomeric membrane complexes (such as GPIb α ·GPIb β ·GPIbIX) that fail to assemble properly are not transported beyond the endoplasmic reticulum and are degraded intracellularly. Thus expression of the (α) gene without simultaneous expression of the (β) and (IX) genes is not expected to result in the isolation of an (α) polypeptide or biologically active forms thereof. (Lopez, J.A. et al., Circulation, 82(4), 597a (1990), Krangel, M.S. et al., Cell, 18, 979-991 (1979), Woods, C.M. et al., Cell, 40, 959-969 (1985), Minami, Y. et al., Proc. Natl. Acad. Sci. USA, 84, 2688-2692 (1987)).

As expected, the 79 kDa polypeptide was not detected in culture medium from pMW1 transformed cells. Instead the major GPIb(α) polypeptide isolated from such medium has an approximate apparent molecular weight of 45 kDa, characteristic of the properly glycosylated amino terminal domain of GPIb α . The presence of this species in the culture medium of pMW1 transformed cells demonstrates that the amino terminal domain of GPIb α can be processed as a secretory protein and reaches structural maturation (A) in the absence of assembly of the other components of the GPIb complex and (B) in spite of the usual proteolysis of the full length GPIb α polypeptide.

It is anticipated, however, that stable cell lines will be found which allow for more substantial expression of the full length polypeptide, and the proper folding and glycosylation thereof. As demonstrated below, the His¹-Ala³⁰² fragment contains sufficient primary sequence information to be assembled into a structure possessing domains of tertiary structure present in native glycoprotein Ib α . Similarly, using procedures well known in the art, and/or following, for example, the methods outlined directly below, a functionally equivalent His¹-Arg²⁹³ receptor fragment (for vWF or thrombins) can be prepared. It is expected that the expression of a polypeptide containing the amino acid sequence from approximately His¹ to approximately Ala³⁰², and additional GPIb α sequence on the carboxy terminal side of Ala³⁰², will

also result in a polypeptide possessing the biological activity of the 45 kDa fragment.

Step 4 - Expression of a His¹-Ala³⁰² GPIb α
Fragment in Stable Mammalian Transformants

5 This example demonstrates conditions under which a DNA sequence encoding the fragment of mature GPIb α polypeptide having an amino terminus at His¹ and a carboxy terminus at residue Ala³⁰² thereof may be expressed in and secreted from cultured mammalian cells.

10 The following section concerns primer directed amplification of DNA. pBluescript KS⁺ containing at its BamHI site the 2161 base pair fragment (nucleotides 503-2664 according to Wenger et al.) was subjected to enzymatic amplification in a polymerase chain reaction according to the
 15 method of Saiki et al., and following generally the procedures described above.

The following oligonucleotides were synthesized by the phosphoramidite method, Sinha et al., using a model 380B automated system, Applied Biosystems, Foster City, CA.

20 Nucleotides are shown using the numbering system of Wenger et al. for the GPIb α gene.

Oligonucleotide (C) (SEQ ID NO: 13)

25 502 525
 ↓ ↓
 5' GGATCCACTCAAGGCTCCCTTGCC 3'
 BamHI

Oligonucleotide (D) (SEQ ID NO: 14)

3' CAG TTC AAG GGG TGG TTT CG 5' ← link with BamHI
 5' gtc aag ttc ccc acc aaa gc 3'
 30 Val Ala
 296 302

(nucleotide positions 1470-1489)

Oligonucleotide (C) is equivalent to nontranscribed (coding) strand DNA. Oligonucleotide (D), shown in capital
 35 letters, is equivalent to transcribed (noncoding) strand DNA. The corresponding coding strand for oligonucleotide (D) is shown 5' → 3' with the encoded amino acids shown by standard three letter designation.

A BamHI linker was added to the amplified double stranded DNA sequence 3' to the partial Ala³⁰² codon thereby completing the codon and enabling the DNA to function as a BamHI insert. Roberts et al., Nature, 265, 82-84 (1977).

5 The amplified fragment was then cloned into the BamHI site within the multiple cloning sequence of the double stranded replicative form of M13mp19 bacteriophage. The ability to isolate a stable single stranded (+) form of the virus is particularly useful to verify the integrity of any
10 cloned sequences therein. See, for example, Messing and Yanish-Perron et al.

Accordingly, the GPIb α DNA insert was completely sequenced using single stranded dideoxy methodology, Sanger et al., utilizing the single stranded (+) form of M13mp19 to
15 confirm that the GPIb α fragment contained the correct coding sequence for the region of GPIb α DNA represented by nucleotides 502 to 1489 and including a codon for the initiating methionine, the remaining 15 residues of the signal peptide and residues 1 to 302 of the amino terminal
20 region of mature GPIb α polypeptide.

Sequencing in M13mp19 established numerous clones having insert orientation at the BamHI site suitable for expression from pCDM8^{neo} plasmid. The GPIb α sequence of one such clone was removed from M13mp19 as an EcoRI (5') - XbaI (3')
25 fragment which was then cloned into the polylinker region of pBluescript KS-. An XhoI (5') - NotI (3') fragment of this second insert was then removed from pBluescript KS- and cloned into pCDM8^{neo}, which had been restricted with Xho and NotI, following the procedures described above used for
30 insertion of pMW1.

Ampicillin sensitive E. coli strain SX-127 cells (Invitrogen, San Diego, CA) were transformed with the resultant ligated DNA mixture following the method of Hanahan, J. Mol. Biol., 166, 557-580 (1983).

35 Plasmids from resultant colonies were characterized by restriction mapping and DNA sequencing to identify colonies which contained the intended insert. One such appropriate plasmid (designated pMW2) was maintained in E. coli strain XS-127, and was selected for mammalian cell transformation

procedures.

Prior to use in transforming mammalian cells, supercoiled plasmids (pMW2) were recovered from host E. coli by the alkaline lysis procedure of Birnboim and Doly followed by CsCl/ethidium bromide equilibrium centrifugation according to the procedure described above. Transformation of CHO-K1 cells also followed the procedure therefor described above for pMW1 plasmid.

10 Preparation of vWF Domains Useful as Components of Bifunctional Antithrombotic Molecules (Examples 3-7)

Example 3 - Expression of a Mutant Cysteine-Free Mature von Willebrand Factor Subunit Fragment Having an Amino Terminus at Residue 441 (Arginine) and a Carboxy Terminus at Residue 733 (Valine)

15 Preparation of a cDNA Clone from pre-pro-von Willebrand Factor mRNA

A cDNA clone encoding the entire von Willebrand factor gene (for the pre-propeptide) was provided by Dr. Dennis Lynch, Dana-Farber Cancer Institute, Boston, MA and was prepared as described in Lynch, D.C. et al., Cell, 41, 49-56 (1985). It had been deemed probable that the size of vWF mRNA would likely exceed that of human 28S type rRNA. Accordingly, total RNA from endothelial cells (the major source of plasma vWF) was sedimented in sucrose gradients, with RNA larger than 28S being selected for construction of a cDNA library.

This enriched fraction was further purified using two separate cycles of poly(u)-Sephadex® chromatography to select for RNA species (mRNA) having 3' polyadenylated ends. Lynch et al., supra, estimated the prevalence of vWF mRNA in this fraction at about 1 in 500, which fraction was used to generate a cDNA library of approximately 60,000 independent recombinants.

To generate the cDNA library, standard techniques were used. The mRNA population was primed using an oligo (dT) primer, and then transcribed with a reverse transcriptase. The RNA strands were then removed by alkaline hydrolysis, leaving cDNA anticoding strands (equivalent to transcribed strands) which were primed by hairpin looping for second strand synthesis using DNA polymerase I. The hairpin loop

was removed with S_1 nuclease and rough ends were repaired with DNA polymerase I.

GC tailing, Maniatis, T. et al., Molecular Cloning, 2nd ed., v.1, p.5.56 (1987), was then used to anneal the cDNA into plasmid vector pBR322. Oligo(dC) tails were added to the cDNA fragments with terminal transferase and were annealed to oligo(dG) tailed pBR322. The plasmids were transformed into ampicillin sensitive E.coli, strain HB101 for propagation. Suitable clones were identified after screening with ^{32}P -labelled cDNA prepared as reverse transcriptase product of immunopurified vWF polysomes. Positive clones were subcloned into pSP64 (Promega Co., Madison, WI).

Primer Directed Amplification of cDNA

cDNA representing the full length pre-pro-vWF gene from pSP64 was subjected to enzymatic amplification in a polymerase chain reaction. Based upon the established nucleotide sequence of the pre pro-vWF gene, Bonthron, D. et al. Nucl. Acids Res., 14(17), 7125-7127 (1986); Mancuso, D. et al., J. of Biological Chemistry, v.264(33), 19514-19527 (1989) oligonucleotides flanking the region of interest (designated #440, SEQ ID NO: 15, and #730, SEQ ID NO: 16) were prepared. All oligonucleotides used herein were synthesized by the phosphoramidite method, Sinha, et al., Tetrahedron Letters, 24, 5843 (1983), using a model 380B automated system, Applied Biosystems, Foster City, CA. Oligonucleotide #440 (SEQ ID NO: 15)

5'ACGAATTC CGG CGT TTT GCC TCA GGA3'
EcoRI Arg₄₄₁ Gly₄₄₆

Oligonucleotide #730 (SEQ ID NO: 16)

3'GG GAC CCC GGG TTC TCC TTG AGG TAC CAT TCGAAG5'
5'cc ctg ggg ccc aag agg aac tcc atg gta agcttc3'
Leu₇₂₅ Met₇₃₂Val₇₃₃HindIII

The oligonucleotides overlap the ends of the coding region for that fragment of the mature vWF subunit which can be produced by digestion with trypsin and which begins with residue 449 (valine) and ends with residue 728 (lysine).

Oligonucleotide #440 corresponds to coding strand DNA (analogous with mRNA) for amino acid positions 441 to 446 and adds an EcoRI restriction site 5' to the codon for amino acid 441. Oligonucleotide 730 corresponds to the non-coding strand (transcribed strand) of mature vWF DNA for amino acids positions 725-733 and adds a HindIII restriction site 3' to the codon for amino acid 733. The coding strand complementary to #730 is shown in lower case letters.

Using the above oligonucleotides with the full length cDNA as template, a cDNA fragment corresponding to mature vWF residues Nos. 441-733, and containing EcoRI and Hind III linkers, was then synthesized in a polymerase chain reaction following the method of Saiki, R.K. et al. Science, 239, 487-491 (1988).

The procedure utilizes a segment of double-stranded vWF cDNA, a subsegment of which is to be amplified, and two single-stranded oligonucleotide primers (in this case oligonucleotides #440, 730) which flank the ends of the subsegment. The primer oligonucleotides (in the presence of a DNA polymerase and deoxyribonucleotide triphosphates) were added in much higher concentrations than the DNA to be amplified.

The vast majority of polynucleotides which accumulate after numerous rounds of denaturation, oligonucleotide annealing, and synthesis, represent the desired double-stranded cDNA subsegment suitable for further amplification by cloning.

For some experiments, cDNA corresponding to the mature vWF fragment beginning at amino acid sequence position 441 and ending at position 733 was prepared and amplified directly from platelet mRNA following the procedure of Newman, P.J. et al. J. Clin. Invest., 82, 739-743 (1988). Primer nucleotides No. 440 and 733 were utilized as before with the resulting cDNA containing EcoRI and HindIII linkers.

35 Insertion of cDNA into M13mp18 Cloning Vehicle

The resultant double stranded von Willebrand factor cDNA corresponding to the amino acid sequence from residue 441 to 733 was then inserted, using EcoRI and HindIII restriction enzymes, into the double stranded replicative form of

bacteriophage M13mp18 which contains a multiple cloning site having compatible EcoRI and HindIII sequences. See Messing, J. Meth. Enzymology, 101, 20-78 (1983); Yanish-Perron, C. et al. Gene, 33, 103-109 (1985). See also Greene, P.J. et al. 5 J. Biol. Chem., 256, 2143-2153 (1981) and Old, R. et al. J. Mol. Biol., 92, 331-339 (1975) for the properties of EcoRI and HindIII restriction enzymes respectively.

M13 series filamentous phages infect male (F factor containing) E.coli strains. The infecting form of the virus 10 is represented by single stranded DNA, the (+) strand, which is converted by host enzymes into a double stranded circular form, containing also the minus (-) strand, which double stranded structure is referred to as the replicative form (RF). The ability to isolate a stable single stranded (+) 15 form of the virus is particularly useful to verify the integrity of any cloned sequences therein.

Accordingly, the vWF cDNA insert was completely sequenced using single-stranded dideoxy methodology (Sanger, F. et al. Proc. Natl. Acad. Sci USA, 74, 5463-5467 (1977)), 20 utilizing the single-stranded (+) form of M13mp18, to confirm that the vWF cDNA fragment contained the correct coding sequence for mature vWF subunit residues 441-733.

Site-Directed Mutagenesis to Replace Cysteine Residues

Cysteine residues 459, 462, 464, 471, 474, 509, and 695, 25 within the mature vWF fragment corresponding to amino acids 441 to 733, were replaced with glycine residues by substitution of glycine codons for cysteine codons in the corresponding cDNA. In order to accomplish this, oligonucleotides (see Sequence Listing ID NOS: 17-20) 30 encompassing the region of each cysteine codon of the vWF cDNA were prepared as non-coding strand (transcribed strand) with the corresponding base substitutions needed to substitute glycine for cysteine. The oligonucleotides used were as follows:

Oligonucleotide #459 (SEQ ID NO: 17)

3'GGA CTC GTG CCG GTC TAA CCG GTG CAA CTA CAA CAG5'
 5'cct gag gac ggc cag att ggc cac ggt gat gtt gtc3'
 Pro Glu His Gly Gln Ile Gly His Gly Asp Val Val
 459 462 464
 5 (simultaneously replacing cysteines 459, 462, 464).

Oligonucleotide #471 (SEQ ID NO: 18)

3'TTG GAG TGG CCA CTT CGG CCG GTC CTC GGC5'
 5'aac ctc acc ggt gaa gcc ggc cag gag ccg3'
 10 Asn Leu Thr Gly Glu Ala Gly Gln Glu Pro
 471 474
 (simultaneously replacing cysteines 471, 474)

Oligonucleotide #509 (SEQ ID NO: 19)

3'CTA AAG ATG CCG TCG TCC G5'
 15 5'gat ttc tac ggc agc agg c3'
 Asp Phe Tyr Gly Ser Arg
 509
 (replacing cysteine 509)

Oligonucleotide #695 (SEQ ID NO: 20)

20 3'TCG ATG GAG CCA CTG GAA CCG5'
 5'agc tac ctc ggt gac ctt gcc3'
 Ser Tyr Leu Gly Asp Leu Ala
 695
 (replacing cysteine 695)

25 Hybridizing oligonucleotides are shown in capital letters and are equivalent to the transcribed strand (non-coding DNA). The equivalent coding strand is shown in lower case letters with the corresponding amino acids shown by standard three letter designation. (for designations see
 30 Table 1)

As elaborated below, cysteines 459, 462 and 464 were replaced simultaneously using oligonucleotide 459. Cysteine residues 471 and 474 were then replaced simultaneously using oligonucleotide 471. Cysteine residues 509 and 695 were then
 35 replaced individually using oligonucleotides 509 and 695 respectively.

The cysteine to glycine cDNA substitutions were accomplished following the procedure of Kunkel, T.A., Proc. Natl. Acad. Sci. USA, 82,488-492 (1985) which procedure

repeats a series of steps for each oligonucleotide and takes advantage of conditions which select against a uracil containing DNA template:

- 5 (A) M13mp18 phage, containing wild type vWF cDNA corresponding to amino acid positions 441 to 733, is grown in an E.coli CJ236 mutant dut⁻ung⁻ strain in a uracil rich medium. Since this E.coli strain is deficient in deoxyuridine triphosphatase (dut⁻), an intracellular pool of dUTP accumulates which competes with dTTP for incorporation into DNA. (see Shlomai, J. et al. J. Biol. Chem., 253(9), 3305-3312 (1978). Viral DNA synthesized under these conditions includes several uracil insertions per viral genome and is stable only in an E.coli strain which is incapable of removing uracil, such as
- 10 (ung⁻) strains which lack uracil glycosylase. Uracil-containing nucleotides are lethal in single stranded (+) M13mp18 DNA in ung⁺ strains due to the creation of abasic sites by uracil glycosylase.
- 15 (B) Single-stranded (+) viral DNA is isolated from culture media in which phage were grown in E.coli strain CJ236 dut⁻ung⁻. The single stranded (+) form of the virus contains the specified vWF cDNA at its multiple cloning site which cDNA is equivalent to the nontranscribed vWF DNA strand.
- 20 (C) Oligonucleotide #459, which contains codon alterations necessary to substitute glycines for cysteines at positions 459, 462 and 464, is then annealed in vitro to single stranded (+) phage DNA.
- 25 Generally, a wide range of
- 30
- 35

oligonucleotide concentrations is suitable in this procedure. Typically 40 ng of oligonucleotide was annealed to 0.5-1.0 μ g M13mp18 phage (+) DNA.

5

(D) All missing sequence of the M13mp18(-) strand is then completed in vitro using T₇ DNA polymerase and T₄ DNA ligase in a dTTP rich environment thereby generating a transcribable vWF cDNA sequence corresponding to amino acid positions 441 to 733 of the mature vWF subunit.

10

15

(E) The double stranded M13mp18 phage, now containing a thymine normal (-) strand and a (+) strand with several uracil substitutions, is transformed into a wild type E.coli XL-1 Blue (Stratagene, La Jolla, CA) strain which contains normal levels of uracil glycosylase and deoxyuridine triphosphatase.

20

25

(F) Uracil glycosylase and other enzymes present in the new host initiate destruction of the uracil-containing (+) strand of the double-strand phages, leading after replication in the host of remaining phage (-) strand DNA to the presence of stable thymine-normal double stranded (RF) DNA which reflects the glycine mutations induced by the oligonucleotide.

30

(G) Steps (A) to (F) of the above process are then repeated for each of oligonucleotides #471, 509 and 695 until each successive cysteine codon of the vWF sequence within the M13mp18 phage has been replaced by a glycine codon.

35

(H) Upon completion of mutagenesis procedures the sequence of the vWF cDNA insert was reconfirmed using the single stranded DNA dideoxy method. (Sanger, F. et al.,

supra).

Construction of Expression Plasmids.

The double stranded vWF cDNA fragment containing 7 site-specific cysteine to glycine mutations is then removed from
5 M13mpl8 phage by treatment with EcoRI and HindIII restriction endonucleases, after which the ends of the fragment are modified with BamHI linkers (Roberts, R.J. et al. Nature, 265, 82-84 (1977)) for cloning into a high efficiency E.coli expression vector. The particular expression vector chosen
10 is plasmid pET-3A, developed by Rosenberg, A.H. et al. Gene, v.56, 125-135, (1987) and which is a pBR322 derivative containing a high efficiency ($\phi 10$) T7 transcription promoter directly adjacent to the BamHI linker site. When containing the above-specified fragment of mutant vWF cDNA, the pET-3A
15 vehicle is referred to as "p7E" or p7E expression plasmid.

A second pET-3A-derived expression plasmid (designated p7D) was constructed containing the identical vWF coding sequence cloned into the plasmid in the opposite orientation. p7D should be unable to express the vWF polypeptide fragment.

20 A third expression plasmid (pJD18) contains wild type "52/48 tryptic vWF fragment" cDNA encoding the vWF amino acid sequence between residues 441 and 733, (with 7 cysteines) in the same pET-3A vector.

The p7E (or p7D and pJD18) expression plasmids were then
25 cloned into an ampicillin sensitive E.coli strain, BL21(DE3), Novagen Co., Madison WI, according to a well established protocol Hanahan, D., J. Mol. Biol., 166, 557-580 (1983). Strain BL21(DE3) is engineered to contain a gene for T7 RNA polymerase so that the vWF insert can be transcribed with
30 high efficiency.

Expression of Mutant vWF Polypeptides

Three separate samples of E.coli strain BL21(DE3) containing respectively p7E, p7D or pJD18 expression plasmids were inoculated into 5-6 ml of 2X-YT growth medium
35 containing 200 μ g/ml of ampicillin, and grown overnight at 37°C to create fully grown cultures. 2X-YT growth medium contains, per liter of water, 10 gm Bacto-tryptone, 10 gm yeast extract and 5 gm NaCl. Five ml of each overnight culture was then inoculated into 500 ml of 2X-YT medium,

again containing 200 µg/ml of ampicillin and grown for 2 hours at 37°C with shaking.

After the 2 hour incubation period, the cultures were induced for protein expression by addition of isopropyl-beta-
5 d-thiogalactopyranoside to a concentration of 5 mM. The incubation was then continued for 3 hours at 37°C.

A high level of expression of vWF polypeptide was obtained with p7E and pJD18 resulting in the generation of cytoplasmic granules or "inclusion bodies" which contain high
10 concentrations of vWF polypeptide in essentially insoluble form. Solubilization of vWF polypeptide was accomplished according to the following procedure. As explained in Example 4, p7E and pJD18 extracts responded very differently to solubilization procedures. See Maniatis, T. et al.,
15 Molecular Cloning, 2nd ed., vol. 3, Sec. 17.37, (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, for a general discussion of the properties of, and successful manipulation strategies for, inclusion bodies.

The cells were harvested by centrifugation at 4000 g for
20 15 minutes in a JA-14 rotor at 4°C. The pelleted cells were washed in 50 ml of ice cold buffer (0.1 M NaCl, 10 mM Tris pH 9.0, 1 mM EDTA) and repelleted by centrifugation at 4000 g at 4°C.

The cell pellets from p7E, p7D and pJD18 cultures were
25 each redissolved in 5 ml of lysing buffer and kept ice-cold for 30 minutes. The lysing buffer comprises a solution of sucrose 25%(w/v), 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM ethylene diaminetetraacetic acid (EDTA), 2 mg/ml lysozyme and 50 mM Tris hydrochloride, adjusted to pH 8.0.

30 After the 30 minute incubation, aliquots of 1.0 Molar MgCl₂ and MnCl₂ were added to make the lysing solution 10 mM in each cation. Sixty µg of DNaseI (Boehringer-Mannheim) was then added and the incubation was continued at room temperature for 30 minutes.

35 Twenty ml of buffer No. 1 (0.2 M NaCl, 2 mM EDTA, and 1% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 1% (w/v) Nonidet 40, and 20 mM Tris hydrochloride, pH 7.5) was then added to the incubation mixture. The insoluble material was pelleted by

centrifugation at 14,000 g (12,000 rpm in a JA-20 rotor) for 30 minutes at 4°C.

The relatively insoluble pelleted material derived from each culture (which contains the desired polypeptides except in the case of p7D) was washed at 25°C in 10 ml of buffer No. 2 (0.5% (w/v) Triton X-100 surfactant, 2 mM EDTA, 0.02 M Tris hydrochloride, pH 7.5) and vortexed extensively. The suspension was centrifuged at 14,000 g for 30 minutes at 4°C and the supernatant was then discarded. The process of resuspension of the pelleted material in buffer No. 2, vortexing and centrifugation was repeated twice.

Each pellet was then washed in 5 ml of buffer No. 3 (0.02 M Tris hydrochloride, pH 7.5, and 2 mM EDTA) at 25°C and vortexed extensively. The suspension was then centrifuged at 4°C for 30 minutes at 14,000 g after which the supernatant was discarded leaving a pellet of inclusion body derived material with a clay-like consistency.

The insoluble pellet was slowly redissolved in an 8 Molar urea solution held at room temperature for 2 hours, after which solubilization was continued overnight at 4°C. The urea-soluble material was extensively dialyzed against a solution of 0.15 M NaCl containing 20 mM Hepes (N-[2-hydroxyethyl]piperazine-N-[2-ethanesulfonic acid]) (pH 7.4) at 4°C.

The solubilized peptide extracts were assayed for purity (Example 4), used in vWF binding inhibition assays, or subject to further purification. Further purification steps should not be delayed and the samples should remain cold.

The cysteine-free vWF polypeptide (comprising subunit positions 441 to 733) constitutes more than 75% of the material solubilized from the inclusion bodies according to the above procedure. Further purification of the cysteine-free mutant vWF polypeptide is accomplished by redialyzing the partially purified peptide extract against 6 M guanidine·HCl, 50 mM Tris·HCl, pH 8.8 followed by dialysis against 6 M urea, 25 mM Tris·HCl, 20 mM KCl, 0.1 mM EDTA, pH 8.0. The extract is then subjected to chromatography on a Q-Sepharose® column (Pharmacia, Uppsala, Sweden). The column was pre-equilibrated with 6 M urea, 25 mM Tris·HCl, 20 mM KCl,

0.1 mM EDTA pH 8.0. Elution of the vWF polypeptide utilized the same buffer except that the concentration of KCl was raised to 250 mM. Polypeptide samples used for further assays were redialyzed against 0.15 M NaCl, 20 mM Hepes, pH 7.4. However, long term storage is best achieved in urea buffer (6 M urea, 25 mM Tris·HCl, 20 mM KCl, 0.1 mM EDTA pH 8.0. Final p7E-vWF polypeptide percent amino acid compositions (by acid hydrolysis) compared closely with values predicted from published sequence information (Bonthron, D. et al. and also Mancuso, D. et al. above in Example 3).

Example 4 - Characterization of the Cysteine-Free Mutant von Willebrand Factor Fragment Produced by Expression Plasmid p7E

Urea-solubilized and dialyzed polypeptides extracted from inclusion bodies of cultures containing expression plasmids p7E, p7D and pJD18 were analyzed using polyacrylamide gel electrophoresis (PAGE) and immunoblotting. SDS-polyacrylamide gel electrophoresis procedures and results are described below.

The purity and nature of the expression plasmid extracts, which had been urea-solubilized and then extensively dialyzed, were first analyzed using the denaturing sodium dodecylsulfate-polyacrylamide gel electrophoresis procedure of Weber, K. et al. J. Biol. Chem., 244, 4406-4412 (1969), as modified by Laemli, U.K. Nature, 227, 680-685 (1970) using an acrylamide concentration of 10%. The resultant gels were stained with Coomassie blue and compared.

The extract from expression plasmid p7E contains as the major component, the mutant von Willebrand factor polypeptide which migrates with an apparent molecular weight of approximately 36,000 Daltons. The polypeptide appears as a single band under both reducing conditions (addition of between 10 and 100 mM dithiothreitol "DTT" to the sample for 5 min at 100°C prior to running the gel in a buffer also containing the same DTT concentration) and nonreducing conditions, which result is consistent with the substitution of glycine residues for all of the cysteine residues therein. No vWF polypeptide could be extracted from host cells

containing p7D expression plasmids as expected from the opposite orientation of the vWF cDNA insert.

The cysteine-containing vWF polypeptide expressed by host cells containing pJD18 plasmids, and which contains the wild type amino acid sequence of the 52/48 fragment, (herein represented by a residue 441 to 733 cloned fragment) behaved differently under reducing and nonreducing conditions of electrophoresis. The wild-type sequence expressed from pJD18 forms intermolecular disulfide bridges resulting in large molecular weight aggregates which are unable to enter the 10% acrylamide gels. After reduction (incubation with 100 mM DTT for 5 min at 100°C), the vWF peptide migrates as a single band with a molecular weight of approximately 38,000.

Example 5 - Expression of a Mutant vWF Fragment of Reduced Cysteine Content Containing a Disulfide-Dependant Conformation

Utilizing the procedures of Example 3, except as modified below, a mutant vWF polypeptide fragment (corresponding to the mature vWF subunit sequence from residue 441 to residue 733) was prepared in which the cysteines at positions 459, 462, 464, 471 and 474 were each replaced by a glycine residue. Cysteine residues were retained at positions 509 and 695, and allowed to form an intrachain disulfide bond.

Site directed mutagenesis was performed only with oligonucleotides No. 459 and 471, thereby substituting glycine codons only at positions 459, 462, 464, 471 and 474. Upon completion of mutagenesis procedures, the sequence of the mutant vWF cDNA was confirmed using the single-stranded dideoxy method.

The double-stranded form of the vWF cDNA insert (containing 5 cysteine to glycine mutations) was then removed from M13mp18 phage by treatment with EcoRI and HindIII restriction endonucleases, modified as in Example 3 with BamHI linkers, and cloned into pET-3A. The pET-3A vehicle so formed is referred to as "p5E" or p5E expression plasmid.

The p5E expression plasmids were then cloned into ampicillin sensitive E.coli strain BL21(DE3), Novagen Co., Madison, WI, according to the procedure of Hanahan, D., J. Mol. Biol., 166, 557-580 (1983). The p5E mutant polypeptide

was expressed from cultures of E.coli BL21(DE3) following the procedure of Example 3 except that solubilization of inclusion body pellet material in the presence of 8 Molar urea need not be continued beyond the initial 2 hour period at room temperature, at which point redissolved material had reached a concentration of 200 µg/ml.

As in Example 3 pertaining to the p7E extracts, final purification of urea-solubilized inclusion body preparations was accomplished by dialysis against 6 M guanidine and urea solutions followed by Q-Sepharose® chromatography.

Example 6 - Preparation of Subfragments of the Residue 441-733 vWF Fragment Useful as a Domain of the Bifunctional Molecules of the Invention

This example is illustrative of the preparation of domains of bifunctional antithrombotic molecules that are derived from the residue 441-733 fragment of vWF subunit. The example is also illustrative of conditions under which such vWF subfragments may be expressed from recombinant bacterial host cells. The subfragments may be expressed also from recombinant eucaryotic cells, for example, by following the general procedures of Example 7. The vWF subfragments are capable of interfering with the interaction of multimeric vWF and platelet GPIIb/IIIa, that is, they have utility as a domain of a bifunctional antithrombotic molecule.

Fusion Polypeptide Subfragments of the Residue 441-733 Domain of vWF Subunit

Mutant (fusion) polypeptides consisting of the residue 441-733 sequence, but lacking either the internal G10 (residues 474-488) or D5 (residues 694-708) region, were created using loopout mutagenesis in M13mp18 phage of restriction fragments of p7E constructs and then tested for antithrombotic activity.

Specifically, p7E plasmids were recovered from cultures of E.coli BL21(DE3) using an alkaline cell lysis procedure, Birnboim, H.C. and Doly, J., Nucleic Acids Research, 7, 1513 (1979) followed by purification by CsCl/ethidium bromide equilibrium centrifugation. An XbaI restriction site exists in p7E plasmid (contributed by the parent pET-3A vector) upstream from the T7 transcription promoter. Accordingly,

the VWF insert (for residues 441-733) was removed as an XbaI-HindIII restriction fragment for loopout mutagenesis (see Example 3) in M13mp18 phage. Loopout of the G10 region or D5 region, respectively, was accomplished using the following oligonucleotides which represent non-coding strand (transcribed strand) DNA. Shown below the two 3' → 5' oligonucleotides are the corresponding coding strands and resultant amino acid sequences.

Oligonucleotide (E) - see SEQ ID NO: 21

10 3' - GAG TGG CCA CTT CGG CAC TCG GGG TGG TGA - 5'
 5' - ctc acc ggt gaa gcc gtg agc ccc acc act - 3'
 Leu Thr Gly Glu Ala Val Ser Pro Thr Thr
 469 470 471 472 473 489 490 491 492 493

15 deletion of G10 binding peptide

Oligonucleotide (F) - see SEQ ID NO: 22

20

3' - CTC TAG CAA TCG ATG CTG TAC CGT GTT CAG - 5'

5' - gag atc gtt agc tac gac atg gca caa gtc -3'

Glu Ile Val Ser Tyr Asp Met Ala Gln Val

689 690 691 692 693 709 710 711 712 713

deletion of D5 binding peptide

DNA sequence analysis was used to confirm that the intended vWF coding sequences were produced. The two
25 mutagenized XbaI-HindIII restriction fragments were then inserted into separate pET-3A plasmids that had been cut with XbaI and HindIII restriction endonuclease and which were thereafter designated p7E/ Δ G10 and p7E/ Δ D5.

The resultant mutant (fusion) vWF polypeptides were then tested for their ability to bind to GPIIb/IIIa. Using an assay dependent on inhibition of the binding of LJ-Ib1 antibody to GPIIb/IIIa in the absence of botrocetin modulator, it was determined that the residue 441-733 fragment, which was expressed from p7E and from which the "G10" peptide sequence was deleted, binds GPIIb/IIIa. The p7E-derived fusion fragment lacking the "D5" peptide sequence did not. However, when the experiments were repeated using botrocetin as a modulator of binding, both of the fused subfragments were effective in inhibiting binding by LJ-Ib1, and hence have utility as domains of bifunctional antithrombotics.

Other in vitro assays which can be used to identify vWF-

derived domains having such antithrombotic activity include inhibition of botrocetin-induced binding of vWF to platelets by the vWF-derived domain, and the inhibition of human platelet agglutination in a system using bovine vWF, but without a modulator such as botrocetin or ristocetin.

vWF-derived Polypeptide
Subfragments Having N-terminal Deletions

Polypeptide subfragments effective as domains of bifunctional antithrombotics have also been prepared which are patterned upon the residue 441-733 vWF subunit fragment, but which contain N-terminal deletions therefrom.

Preparation of such polypeptides was accomplished using loopout mutagenesis in M13mp18 phage of the XbaI-HindIII restriction fragment from p5E expression plasmid. Thus, the vWF encoding sequence (p5E) encoded cysteine for residue positions 509 and 695 and glycine at residue positions 459, 462, 464, 471 and 474. p7E sequence is also useful for expression of such antithrombotic domains. Antithrombotic polypeptide domains equivalent to those expressed from p7E constructs can be made by reduction and alkylation of cysteine residues otherwise contained therein.

The design of oligonucleotides used to create N-terminal deletions in the vWF subunit fragment made reference to DNA sequence of the pET-3A vector that is upstream (5') from the codon encoding vWF residue 441. Expression of the residue 441-733 fragment as an EcoRI-HindIII insert (with both 5' and 3' ends thereof modified by BamHI linkers, Example 3) in pET-3A involves expression also of a twenty residue amino acid sequence (SEQ ID NO: 23) that remains attached to the amino terminal of the vWF fragment. This sequence, as shown below, is encoded by vector DNA downstream from the T7 promoter site but does not affect adversely the therapeutic activity of the vWF polypeptide.

initiation codon
 ↓
 Met Ala Ser Met Thr Gly Gly Gln Gln Met
 Gly Arg Gly Ser Pro Gly Leu Gln Glu Phe Arg₄₄₁
 ↑
 from EcoRI

It is noted that the EcoRI-encoding sequence (Glu-Phe)

survived modification with a BamHI linker in the T₄-DNA ligase procedure (Example 3) in this particular case. The corresponding pET-3A vector coding sequence located upstream from the initiating methionine and residue 441 (arginine) is as follows.

Oligonucleotide (G) - see SEQ ID NO: 24

5' - GAA GGA GAT ATA CAT ATG GCT AGC . . .
Met Ala Ser

Accordingly, generation of N-terminal deletions was accomplished using loopout mutagenesis with a hybridizing oligonucleotide that encodes sequence from the vector (ending at the initiating methionine) and then the intended N-terminal region of the new vWF polypeptide.

Representative of the oligonucleotides necessary for the preparation of the therapeutic polypeptides is oligonucleotide H (SEQ ID NO: 25) that corresponds to non-coding strand (transcribed strand) DNA. Shown below this oligonucleotide are the corresponding coding strand and resultant amino acids.

3' - CCT CTA TAT GTA TAC GTC CTC GGC CCT CCG - 5'
gga gat ata cat atg cag gag ccg gga ggc
Met Gln Glu Pro Gly Gly
474' 475 476 477 478 479

Representative of vWF subfragments reflecting such N-terminal deletions are Met·Gln⁴⁷⁵ to Val⁷³³ (SEQ ID NO: 26), Met·Thr⁴⁹² to Val⁷³³, and Met·Tyr⁵⁰⁸ to Val⁷³³. Such vWF subfragments (and other species having terminal deletion of any subsets of the vWF residue 441-508 sequence) have anti-thrombotic therapeutic activity. These polypeptides can present also the cysteine 509-695 loop when expressed from p5E constructs.

vWF Subfragments Having C-Terminal Deletions

The procedure used to express recombinant bacterial polypeptides using pET-3A vectors results in polypeptides that comprise also a series of amino acids on the C-terminal side of Val⁷³³, the additional residues arising from translation of vector sequence (see SEQ ID NO: 27 below).

Specifically, residue 441-733 fragments expressed from p5E (or p7E) constructs contain also 22 residues fused to the C-terminal side of residue 733 (valine) resulting from the

expression of vector sequence prior to the first vector stop codon. This additional vector sequence does not affect adversely the therapeutic utility of the resultant polypeptide. However, if desired, other DNA expression constructs (targeting for either the added amino or carboxy terminal vector sequences) could be designed to avoid the additional sequences.

This pET-3A vector sequence, which reflects also modification (Example 3) of the HindIII site of the EcoRI-HindIII fragment by a BamHI linker, is (SEQ ID NO: 27):

Val Ser Ser Asp Pro Ala Ala Asn Lys Ala
733

Arg Lys Glu Ala Glu Leu Ala Ala Ala Thr

Ala Glu Gln *
15 ↑
 stop codon

In order to prepare an appropriate encoding DNA sequence for vWF polypeptides having C-terminal deletions, loopout mutagenesis was performed in p5E using hybridizing oligonucleotides patterned on non-coding strand DNA. To prepare a polypeptide (using the polypeptide ending at residue Asp⁷⁰⁹ as an example), a hybridizing oligonucleotide was created encoding vWF subunit sequence (for example, from residue 706 to 713) that included also between certain codons thereof (for example, codon 709 and codon 710) the stop codon/reading frame shift sequence 3' - ACT·ACT·T - 5'.

Accordingly, vWF-derived polypeptide subfragments useful in the practice of the invention were generated that have C-terminal deletions and which terminate at residues 709 (SEQ ID NO: 28), 704, 700 and 696 respectively.

Example 7 - Expression of vWF Fragments in Eucaryotic Host Cells

Fragments of von Willebrand factor subunit useful as domains of the bifunctional antithrombotic molecules of the invention may be prepared also from recombinant eucaryotic host cells. vWF-derived polypeptides can be expressed in such host cells, or secreted therefrom if appropriate DNA constructs are used containing signal peptide encoding sequence.

Procedures necessary for the expression of the Arg⁴⁴¹ to Asn⁷³⁰ fragment of mature vWF subunit from mammalian host cells are described in the publication Azuma, H. et al., Independent Assembly and Secretion of a Dimeric Adhesive

5 Domain of von Willebrand Factor Containing the Glycoprotein
Ib-Binding Site, J. Biol. Chem., 266(19), 12342-12347 (1991).
It should be noted that this recombinantly produced molecule
intrinsically self-assembles through intermolecular disulfide
bond formation into a dimer (116 kDa) of the 52/48 kDa
10 residue 441-730 polypeptide domain, duplicating its role in
the final structure of vWF as isolated from the blood.

Production of monomeric residue 441-730 polypeptides requires inactivation, deletion or replacement of cysteine residues 459, 462 and 464 thereof (the interchain disulfide contacts). This may be accomplished effectively using site-directed mutagenesis of the vWF construct when contained in an appropriate cloning vehicle, such as M13mp18, using procedures well known in the art. An appropriate oligonucleotide for mutagenesis is as follows.

20 Oligonucleotide (I) - see SEQ ID NO: 29

3' - GGACTCGTGCCGGTCTAACCGGTGCCACTACAACAG - 5'

5' - cctgagcacg^{Gly₄₅₉}ccagattg^{Gly₄₆₂}ccacggtgatg^{Gly₄₆₄}tgttc - 3'

The hybridizing oligonucleotide is shown (3' → 5') in capital letters and is equivalent to transcribed strand (non-coding strand DNA). Underlined letters indicate the single base mutations for the mutant codons. The equivalent coding strand is shown in lower case letters with the corresponding glycine substitutions identified by three letter designation.

30 Example 8 - Determination of the variable region
primary amino acid sequences of LJ-Ib10

Typically, antibody molecules are tetrameric immunoglobulins consisting of two identical light (L) and two identical heavy (H) polypeptide chains. The N-terminal region of each chain, designated V_L or V_H , is usually 110 amino acid residues long and is composed of 3 hypervariable or complementarity-determining regions (CDR) and 4 less variable or framework (FR) regions. The N-terminal region of the light and heavy chain variable regions usually consists of four FR sequences interspersed by 3 CDR sequences, FR1-

CDR1-FR2-CDR2-FR3-CDR3-FR4 (in amino to carboxyl order). Studies on the three-dimensional structure of antibodies have demonstrated that the conformation of the antibody combining sites is determined by the amino acid residues of the CDR
5 (Amit, A.G. et al., Science, 233, 747-758 (1986)). Thus, the CDR sequences provide the unique characteristic features of the antibody, whereas the FR regions provide the structural scaffolding that aligns the CDR.

An ever-expanding database of mouse V_H and V_L chain
10 sequences (Kabat, E.A. et al., Sequences of Proteins of Immunological Interest, U.S. Public Health Service, Bethesda, MD (1987)) allows the design of oligonucleotide primers that can be exploited for PCR-cloning of cDNAs encoding V_H and V_L polypeptides (Jones, S.T. and Bendig, M.M., Bio/Technology,
15 9, 88-89 (1991)). Using such an approach the V_H and V_L coding sequences for LJ-Ib10 can be cloned. Following procedures established in the art, the primary amino acid sequence subdomains that provide LJ-Ib10 with its unique properties can be determined.

20 Hybridoma cells secreting LJ-Ib10 can be expanded to obtain approximately 10^8 cells. The cells are then resuspended in a lysis buffer composed of 4 M guanidine isothiocyanate, 20 mM sodium acetate (pH 5.2), 0.1 mM dithiothreitol, and 0.5% n-lauryl sarcosine. After
25 resuspending the cells, the lysate is passed 3-4 times through a syringe containing a 20 gauge needle to reduce the viscosity of the lysate. The sheared lysate is then layered on top of a 5.7 M cesium chloride cushion and centrifuged for 20 hours at approximately 100,000 x g. The principle of the
30 procedure (see Glisin, V. et al., Biochemistry, 13, 2633 (1974)) is that total RNA when subjected to centrifugation will pellet through the cesium chloride cushion since it represents the most dense biomolecule of the cell.

The total RNA can then be subjected to a first strand
35 cDNA reaction using Moloney murine leukemia virus reverse transcriptase using the recommended assay conditions for the enzyme. The first strand reaction is then primed with oligonucleotides designated VH1FOR (specific for FR4 of heavy chain) and Vk1FOR (κ -chain variable primer analogous to FR4

of the light chain) shown below:

SEQ ID NO: 30

VH1FOR 5'- TGAGGAGACG GTGACCGTGG KpnI TACCTTGGCC CCAG -3'

5 SEQ ID NO: 31

Vκ1FOR 5'- GTTAGATCTC BglII CAGCTTGGTC CC -3'

These oligonucleotides were designed to represent non-coding strand sequence for the indicated regions but are not necessarily an exact match for the noncoding strand of all light and heavy chain genes. The oligonucleotides along with others suitable for generating an antibody cDNA have been described by Orlandi, R. et al., Proc. Natl. Acad. Sci. USA, 86, 3844-3847 (1989); Chiang, Y.L. et al., BioTechniques, 7, 360-366 (1989); Larrick, J.W. et al., Bio/Technology, 7, 934-938 (1989); Loh, E.Y. et al., Science, 243, 217-219 (1989); Sastry, L. et al., Proc. Natl. Acad. Sci. USA, 86, 5728-5732 (1989); Ward, E.S. et al., Nature, 341, 544-546 (1989); Huse, W.D. et al., Science, 246, 1275-1281 (1989); and Jones, S.T. and Bendig, M.M., Bio/Technology, 9, 88-89 (1991). The oligonucleotides represent noncoding strand sequence and will anneal to the corresponding coding sequence present within the specific mRNA species responsible for the generation of LJ-Ib10. The conserved sequence among immunoglobulin gene subsegments allows this PCR-based strategy to be successful.

Aliquots of the first-strand reaction can then be subjected to a polymerase chain reaction using Taq DNA polymerase and two more oligonucleotides representing coding sequence for the signal peptide sequence and FR1 region. Two representative oligonucleotides are shown below:

SEQ ID NO: 32

VH1BACK 5'- AGGT(C/G) (A/C)A(A/C)CT PstI CCAG(C/G)AGTC(A/T) GG -3'

35 SEQ ID NO: 33

Vκ4 5'- PstI TTCTGCAGAT ATTGTGCTAA CTCAGTCTCC -3'

Practically, it is recognized in the art that significant variability exists at the amino-terminus of antibody variable regions and this necessitates the testing

of several different oligonucleotides to insure amplification of a cDNA fragment encoding an antibody (a list of coding strand oligonucleotides that have been successfully used for different monoclonal antibodies is provided by Jones, S.T.

5 and Bendig, M.M., Bio/Technology, 9, 88-89 (1991).

The amplified fragments can then be cloned into M13 vectors as PstI/KpnI fragments (heavy chain) and PstI/BglII fragments (light chain). The nucleotide sequence of the inserts would be determined by DNA sequence analysis. It is
10 known that the hybridoma cells secreting LJ-Ib10 also secrete an antibody designated MOPC-21. The myeloma cell line used in the initial fusion to generate the LJ-Ib10-secreting hybridoma secretes the MOPC-21 immunoglobulin. The sequence of MOPC-21 is known (Kabat, E.A. et al., Sequences of
15 Proteins of Immunological Interest, U.S. Public Health Service, Bethesda, MD (1987)), and although it may be cloned and sequenced using the described strategy because of its known cDNA and primary amino acid sequence, its presence is not a technical problem.

20 Expression of a recombinant LJ-Ib10 variable region domain

Once the primary sequence of LJ-Ib10 variable regions are determined, expression constructs that synthesize light-chain/heavy-chain fusions can be created. For this purpose E.coli expression systems are preferred as they afford a
25 number of advantages in constructing recombinant antibodies (Pluckthun, A., Bio/Technology, 9, 545-551 (1991)). Although a number of different strategies exist for expressing antibodies in prokaryotic systems, in general, they all focus on methods resulting in linked light and heavy chain variable
30 regions (F_v fragments) that fold into a conformation mimicking the variable region of the native antibody.

A F_{ab} fragment can be prepared easily by proteolytic cleavage of an antibody. F_{ab} fragments have the same antigen binding capacity as the whole antibody. Generating a F_{ab}
35 fragment is well exploited technique to reduce the size of an immunoglobulin and to avoid numerous possible complications (see above) associated with expression of a full size antibody construct. However, only half of the F_{ab} fragment is required for antigen binding because it is composed of a

constant region (approximately one-half of the F_{ab}) and two variable region binding arms. An F_v fragment represents generally the smallest antibody fragment that still contains an antigen binding site. It is essentially composed of the 4 framework regions interspersed with the 3 CDR for both a heavy and a light chain in combination. F_v fragments are difficult to prepare by proteolysis, but have become the structural framework for synthesizing recombinant antibodies using genetic techniques.

There is below described a strategy to generate a recombinant F_v fragment of LJ-Ib10, although a number of other different expression strategies exist and are summarized by Pluckthun, A., Bio/Technology, 9, 545-551 (1991). According to the proposed strategy, there is first generated a single-chain F_v polypeptide sequence (consisting of the light chain variable region only), a linker polypeptide sequence, and then the heavy chain variable region. Site-directed mutagenesis strategies can be used to fuse the cDNA coding sequences for each region in a single open reading frame that will produce the F_v in the form of single-chain polypeptide.

The following genetic manipulations represent an example of such a strategy. An M13 construct containing the coding sequence for heavy chain variable region (as a KpnI-PstI fragment) can be mutated so that it is flanked by the recognition sequence for the restriction enzyme BamHI. The mutagenesis procedure would use established technology as described by Kunkel, T.A. et al., Methods in Enzymology, 154, 367-383 (1987), see also Example 3 herein. The PstI-BamHI fragment containing the coding sequence for the light chain variable region would be excised from the M13 construct and cloned as a PstI-BamHI fragment into pBS/KS- (Stratagene, La Jolla, CA). After isolation of recombinant plasmids containing the light chain cDNA, the BamHI fragment containing the heavy chain sequence can be cloned into the recombinant plasmid containing the light chain sequence. A representative recombinant plasmid would then be identified through restriction enzyme analysis and DNA sequence analysis that contains the light-heavy chain cDNA fragment oriented in

a manner that would, in a 5'→3' coding direction, contain coding sequence for light chain variable sequence followed by coding sequence for heavy chain variable sequence.

Further mutagenesis would be performed to create a

5 "linker" or "spacer" sequence generating the single open reading frame for the light and heavy chain polypeptides separated by a polypeptide sequence that facilitates the in vitro association of light and heavy chains. A number of different "linkers" have been described (Huston, J.S. et al.,

10 Proc. Natl. Acad. Sci. USA, 85, 5879-5883 (1988); Glockshuber, R. et al., Biochemistry, 29, 1362-1367 (1990); and Holvoet, P. et al., J. Biol. Chem., 266, 19717-19724 (1991)) and a representative linker is (Gly-Gly-Gly-Gly-Ser)₃. A final mutagenesis in order to make the composite

15 fragment compatible with cloning in an E. coli expression vector such as pET3a (Studier, F.W. and Moffatt, B.A., J. Mol. Biol., 189, 113-130 (1986)) can then be performed. The pET3a vector allows the isolation of single-chain F_v fragments from within inclusion bodies, and their subsequent

20 purification uses established procedures (see Example 3).

Characterizations of a recombinant LJ-Ib10 F_v could include: (1) reactivity with GPIIb/IIIa antigen either on the platelet surface or using a recombinant antigen that contains the epitope of LJ-Ib10 (Murata, M. et al., J. Biol. Chem. 9, 25 266, 15474-15480 (1991)) or (2) the ability of the recombinant LJ-Ib10 F_v polypeptide to inhibit the binding of [¹²⁵I]LJ-Ib10 to platelets.

A bifunctional inhibitor molecule of
vWF and α-thrombin binding to platelet GPIIb/IIIa

30 There can be prepared a chimeric cDNA fragment that comprises coding sequence for a vWF fragment that contains the binding site thereof for GPIIb/IIIa fused to the coding sequence for the "single-chain" LJ-Ib10 F_v described above. The cloning strategy used herein is similar to that used to

35 create the single-chain light and heavy chain fusion construct of LJ-Ib10. Using generally recognized mutagenesis strategies, each fragment can be modified to generate the following elements in a 5' to 3' coding direction: sequence for a vWF fragment that contains the GPIIb binding site, as

example residues 441-728; a "linker" or "spacer" composed of Gly codons to insure flexibility within the linker polypeptide; the coding sequence for the F₁ LJ-Ib10 fragment. The mutagenesis can be performed on the coding sequence of

5 LJ-Ib10 F₁ within the pBS/KS- construct described above. The mutagenesis for the vWF fragment can be performed on constructs, such as that previously described (see Example 3).

The exact modifications to achieve the resultant

10 expression fragment will depend upon the restriction sites present within each component. Each fragment can be altered to contain a restriction recognition sequence for an enzyme recognizing an eight-base DNA sequence, such as NotI, SfiI and PacI. In theory, the eight base sequence will occur once

15 every 65,536 nucleotides ($1/4^8$) and these restriction enzymes are commercially available from New England Bio-Labs (Beverly, MA). The probability that two fragments ranging in size from 600-900 base pairs would contain the restriction recognition sequence of all three enzyme sequences is

20 extremely low. Other mutagenesis strategies could involve the elimination of recognition sequences within the fragments without altering the translated amino acid sequence (taking advantage of the degenerate nature of the genetic code) to allow a ligation of the coding sequences.

25 The final chimeric cDNA fragment will be cloned into pET3a for the preparation of inclusion bodies (see, for example, Example 3) and subsequent isolation of the bifunctional recombinant fragment.

A chimeric molecule consisting of an inhibitor of plasma

30 vWF binding to GPIIb α and for binding of α -thrombin to GPIIb α

Using synthetic peptides the epitope of LJ-Ib10 has been localized to be within GPIIb α residues 237-291 suggesting, since the epitope of LJ-Ib10 coincides (at least in part) with the α -thrombin binding site, that the GPIIb α amino acid

35 residue sequence 237-291 contains the high affinity binding site for thrombin. Synthetic peptides have confirmed this speculation and have identified a peptide composed of approximately residues 271-285 that inhibits the binding of α -thrombin to GPIIb α .

Another type of bifunctional molecule that can simultaneously bind GPIb α and α -thrombin is hereafter proposed. This molecule will be composed of the polypeptide that mimics the GPIb binding site within the vWF (a vWF polypeptide fragment) fused to coding sequence for GPIb α residues 271-285. The vWF fragment construct will be subjected to site-directed mutagenesis (Example 3) to add the indicated GPIb α coding sequence. An oligonucleotide can then be synthesized that contains GPIb α coding sequence for residues 271-285 flanked by sequence present in an M13 construct that contains the coding sequence for a vWF fragment 441-728. The mutagenesis strategy can be employed to insert the GPIb α peptide sequence at the carboxyl-terminal region of the vWF fragment although insertion at the amino terminus is equally possible. An additional "linker" peptide sequence will be included to prevent any structural constraint that might be imposed by the vWF fragment on the GPIb α sequence, or vice-versa.

Deposit of Hybridomas and Strains
Useful in Practicing the Invention

A deposit of biologically pure culture of the following hybridomas/strains was made under the Budapest Treaty with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland. The accession numbers indicated were assigned after successful viability testing, and the requisite fees were paid.

Access to said cultures will be available during pendency of the patent application to one determined by the Commissioner of the United States Patent and Trademark Office to be entitled thereto under 37 C.F.R. §1.14 and 35 U.S.C. §122, or if and when such access is required by the Budapest Treaty. All restriction on availability of said cultures to the public will be irrevocably removed upon the granting of a patent based upon the application and said cultures will remain permanently available for a term of at least five years after the most recent request for the furnishing of a sample and in any case for a period of at least 30 years after the date of the deposit. Should a culture become nonviable or be inadvertantly destroyed, it will be replaced

with a viable culture of the same taxonomic description.

| <u>Hybridomas or Strains</u> | <u>ATCC No.</u> | <u>Deposit Date</u> |
|---|-----------------|---------------------|
| Murine hybridoma LJ-Ib10 (TSRI 138.5) | ATCC HB10939 | 12/3/91 |
| 5 Murine hybridoma LJ-Ib1 (TSRI 138.5) | ATCC HB10940 | 12/3/91 |
| <u>E. coli</u> p5E BL21 (DE3) 96.3 | ATCC 68406 | 9/19/90 |
| <u>E. coli</u> XS127 96.4 | ATCC 68407 | 9/19/90 |

SEQUENCE LISTING

NUCLEOTIDE AND AMINO ACID SEQUENCE DISCLOSURE

PURSUANT TO 37 CFR §1.821(c).

(1) GENERAL INFORMATION:

5 (i) APPLICANT: Ruggeri, Zaverio M.

Ware, Jerry L.

DeMarco, Luigi

Mazzucato, Mario, inventors

on behalf of The Scripps Research

10 Institute

(ii) TITLE OF INVENTION: Bifunctional Antithrombotic
Molecules and Antithrombotic Polypeptides

(iii) NUMBER OF SEQUENCES: 34

(iv) CORRESPONDENCE ADDRESS:

15 (A) ADDRESSEE: The Scripps Research Institute

(B) STREET: 10666 North Torrey Pines Road

(C) CITY: La Jolla

(D) STATE: California

(E) COUNTRY: USA

20 (F) ZIP: 92037

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette-5.25 inch, 1.2 Mb

(B) COMPUTER: AST Bravo IBM PC comp. (386SX)

(C) OPERATING SYSTEM: MS DOS version 3.2

25 (D) SOFTWARE: WordPerfect 5.1 conv. to ASCII

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: Express Mail Label
No. TB122053965US

(B) FILING DATE: 11-Dec-1992

30 (C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA: This appl. is a c-i-p of

(A) APPLICATION NUMBER: 07/613,004

(B) FILING DATE: 13-Nov-1990

(vii) PRIOR APPLICATION DATA: This appl. is a c-i-p of

35 (A) APPLICATION NUMBER: 07/613,083

(B) FILING DATE: 14-Nov-1990

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Barron, Alexis

(B) REGISTRATION NUMBER: 22,702

5 (C) REFERENCE/DOCKET NUMBER: P16,569-E PCT

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (215) 923-4466

(B) TELEFAX: (215) 923-2189

(2) INFORMATION FOR SEQ ID NO: 1:

10 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4

(B) TYPE: Amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: Linear

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Arg Gly Asp Ser

4

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 21

(B) TYPE: Amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: Linear

(ix) FEATURE:

25 (B) LOCATION: 265-285

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Pro Thr Leu Gly Asp Glu Gly Asp Thr Asp
1 5 10

30 Leu Tyr Asp Tyr Tyr Pro Glu Glu Asp Thr Glu
15 20

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15
(B) TYPE: Amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: Linear

(ix) FEATURE:

- (B) LOCATION: 271-285

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

10 Gly Asp Thr Asp Leu Tyr Asp Tyr Tyr Pro
1 5 10
Glu Glu Asp Thr Glu
15

(2) INFORMATION FOR SEQ ID NO: 4:

15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18
(B) TYPE: Amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: Linear

20 (ix) FEATURE:

- (B) LOCATION: 236-253

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

25 Val Lys Ala Met Thr Ser Asn Val Ala Ser
1 5 10
Val Gln Cys Asp Asn Ser Asp Lys
15

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15

(B) TYPE: Amino acid

5 (C) STRANDEDNESS:

(D) TOPOLOGY: Linear

(ix) FEATURE:

(B) LOCATION: 265-279

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

10 Pro Thr Leu Gly Asp Glu Gly Asp Thr Asp
 1 5 10

 Leu Tyr Asp Tyr Tyr
 15

(2) INFORMATION FOR SEQ ID NO: 6:

15 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15

(B) TYPE: Amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: Linear

20 (ix) FEATURE:

(B) LOCATION: 281-295

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

25 Glu Glu Asp Thr Glu Gly Asp Lys Val Arg
 1 5 10
 Ala Thr Arg Thr Val
 15

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5

(B) TYPE: Amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: Linear

(ix) FEATURE:

(B) LOCATION: 271-275

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

10 Gly Asp Thr Asp Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25

(B) TYPE: Amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: Linear

(ix) FEATURE:

(B) LOCATION: 216-240

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Phe Arg Arg Trp Leu Gln Asp Asn Ala Glu
1 5 10

Asn Val Tyr Val Trp Lys Gln Gly Val Asp
15 20

25 Val Lys Ala Met Thr
25

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28
(B) TYPE: Amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: Linear

(ix) FEATURE:

- (B) LOCATION: 235-262

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

10 Asp Val Lys Ala Met Thr Ser Asn Val Ala
1 5 10
Ser Val Gln Cys Asp Asn Ser Asp Lys Phe
15 20
15 Pro Val Tyr Lys Tyr Pro Gly Lys
25

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14
(B) TYPE: Amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: Linear

(ix) FEATURE:

- (B) LOCATION: 269-282

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

25 Asp Glu Gly Asp Thr Asp Leu Tyr Asp Tyr
1 5 10
Tyr Pro Glu Glu

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 31
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

10 G AAT CTG ACA GCG CTG CCT CCA GAC CTG CCG 31
Asn Pro
21 30

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 20
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

20 CAG GGC ACC AAG AGG CAG CG 20
Leu Leu
120 115

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 24
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GGATCCACTC AAGGCTCCCT TGCC

24

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

5

(A) LENGTH: 20

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: single stranded

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

10

GC TTT GGT GGG GAA CTT GAC
Lys Val
301 296

20

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

15

(A) LENGTH: 26

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: single stranded

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

20

ACGAATTC CGG CGT TTT GCC TCA GGA

26

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

25

(A) LENGTH: 35 base pairs

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: single stranded

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GAAGCT TAC CAT GGA GTT CCT CTT GGG CCC CAG GG 35

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

5

(A) LENGTH: 36 base pairs

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: single stranded

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

10

GAC AAC ATC AAC GTG GCC AAT CTG GCC GTG CTC AGG 36

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

15

(A) LENGTH: 30 base pairs

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: single stranded

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

CGG CTC CTG GCC GGC TTC ACC GGT GAG GTT 30

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: Nucleic acid
- 5 (C) STRANDEDNESS: single stranded
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

G CCT GCT GCC GTA GAA ATC 19

(2) INFORMATION FOR SEQ ID NO: 20:

10 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: Nucleic acid
- (C) STRANDEDNESS: single stranded
- (D) TOPOLOGY: Linear

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

GGC AAG GTC ACC GAG GTA GCT 21

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- 20 (B) TYPE: Nucleic acid
- (C) STRANDEDNESS: single stranded
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

AGT GGT GGG GCT CAC GGC TTC ACC GGT GAG 30

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 30 base pairs
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GAC TTG TGC CAT GTC GTA GCT AAC GAT CTC 30

(2) INFORMATION FOR SEQ ID NO: 23:

10 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21
(B) TYPE: Amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: Linear

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

Met Ala Ser Met Thr Gly Gly Gln Gln Met
5 10

Gly Arg Gly Ser Pro Gly Leu Gln Glu Phe Arg
15 20

20 (2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 24 base pairs
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

GAA GGA GAT ATA CAT ATG GCT AGC

24

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 30 base pairs
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

10 GCC TCC CGG CTC CTG CAT ATG TAT ATC TCC

30

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 260
(B) TYPE: Amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: Linear

(ix) FEATURE:

(B) LOCATION: 474'-733

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

20 Met Gln Glu Pro Gly Gly Leu
1 5
Val Val Pro Pro Thr Asp Ala Pro Val Ser
10 15
Pro Thr Thr Leu Tyr Val Glu Asp Ile Ser
25 20 25
Glu Pro Pro Leu His Asp Phe Tyr Cys Ser
30 35

Arg Leu Leu Asp Leu Val Phe Leu Leu Asp
 40 45

Gly Ser Ser Arg Leu Ser Glu Ala Glu Phe
 50 55

5 Glu Val Leu Lys Ala Phe Val Val Asp Met
 60 65

Met Glu Arg Leu Arg Ile Ser Gln Lys Trp
 70 75

10 Val Arg Val Ala Val Val Glu Tyr His Asp
 80 85

Gly Ser His Ala Tyr Ile Gly Leu Lys Asp
 90 95

Arg Lys Arg Pro Ser Glu Leu Arg Arg Ile
 100 105

15 Ala Ser Gln Val Lys Tyr Ala Gly Ser Gln
 110 115

Val Ala Ser Thr Ser Glu Val Leu Lys Tyr
 120 125

20 Thr Leu Phe Gln Ile Phe Ser Lys Ile Asp
 130 135

Arg Pro Glu Ala Ser Arg Ile Ala Leu Leu
 140 145

Leu Met Ala Ser Gln Glu Pro Gln Arg Met
 150 155

25 Ser Arg Asn Phe Val Arg Tyr Val Gln Gly
 160 165

Leu Lys Lys Lys Lys Val Ile Val Ile Pro
 170 175

30 Val Gly Ile Gly Pro His Ala Asn Leu Lys
 180 185

Gln Ile Arg Leu Ile Glu Lys Gln Ala Pro
 190 195

Glu Asn Lys Ala Phe Val Leu Ser Ser Val
 200 205

Asp Glu Leu Glu Gln Gln Arg Asp Glu Ile
 210 215
 Val Ser Tyr Leu Cys Asp Leu Ala Pro Glu
 220 225
 5 Ala Pro Pro Pro Thr Leu Pro Pro His Met
 230 235
 Ala Gln Val Thr Val Gly Pro Gly Leu Leu
 240 245
 10 Gly Val Ser Thr Leu Gly Pro Lys Arg Asn
 250 255
 Ser Met Val
 260

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 23
 (B) TYPE: Amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

20 Val Ser Ser Asp Pro Ala Ala Asn Lys Ala
 5 10
 Arg Lys Glu Ala Glu Leu Ala Ala Ala Thr
 15 20
 25 Ala Glu Gln

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 269
 (B) TYPE: Amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: Linear

(ix) FEATURE:

(B) LOCATION: 441-709

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

| | | |
|----|---|-----|
| 5 | Arg Arg Phe Ala Ser Gly Lys Lys Val Thr | 10 |
| | 5 | |
| | Leu Asn Pro Ser Asp Pro Glu His Cys Gln | 20 |
| | 15 | |
| | Ile Cys His Cys Asp Val Val Asn Leu Thr | 30 |
| | 25 | |
| 10 | Cys Glu Ala Cys Gln Glu Pro Gly Gly Leu | 40 |
| | 35 | |
| | Val Val Pro Pro Thr Asp Ala Pro Val Ser | 50 |
| | 45 | |
| 15 | Pro Thr Thr Leu Tyr Val Glu Asp Ile Ser | 60 |
| | 55 | |
| | Glu Pro Pro Leu His Asp Phe Tyr Cys Ser | 70 |
| | 65 | |
| | Arg Leu Leu Asp Leu Val Phe Leu Leu Asp | 80 |
| | 75 | |
| 20 | Gly Ser Ser Arg Leu Ser Glu Ala Glu Phe | 90 |
| | 85 | |
| | Glu Val Leu Lys Ala Phe Val Val Asp Met | 100 |
| | 95 | |
| 25 | Met Glu Arg Leu Arg Ile Ser Gln Lys Trp | 110 |
| | 105 | |
| | Val Arg Val Ala Val Val Glu Tyr His Asp | 120 |
| | 115 | |
| | Gly Ser His Ala Tyr Ile Gly Leu Lys Asp | 130 |
| | 125 | |
| 30 | Arg Lys Arg Pro Ser Glu Leu Arg Arg Ile | 140 |
| | 135 | |
| | Ala Ser Gln Val Lys Tyr Ala Gly Ser Gln | 150 |
| | 145 | |
| 35 | Val Ala Ser Thr Ser Glu Val Leu Lys Tyr | 160 |
| | 155 | |

| | | | | | | | | | | | |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|
| | Thr | Leu | Phe | Gln | Ile | Phe | Ser | Lys | Ile | Asp | |
| | | | | | 165 | | | | | 170 | |
| | Arg | Pro | Glu | Ala | Ser | Arg | Ile | Ala | Leu | Leu | |
| | | | | | 175 | | | | | 180 | |
| 5 | Leu | Met | Ala | Ser | Gln | Glu | Pro | Gln | Arg | Met | |
| | | | | | 185 | | | | | 190 | |
| | Ser | Arg | Asn | Phe | Val | Arg | Tyr | Val | Gln | Gly | |
| | | | | | 195 | | | | | 200 | |
| 10 | Leu | Lys | Lys | Lys | Lys | Val | Ile | Val | Ile | Pro | |
| | | | | | 205 | | | | | 210 | |
| | Val | Gly | Ile | Gly | Pro | His | Ala | Asn | Leu | Lys | |
| | | | | | 215 | | | | | 220 | |
| | Gln | Ile | Arg | Leu | Ile | Glu | Lys | Gln | Ala | Pro | |
| | | | | | 225 | | | | | 230 | |
| 15 | Glu | Asn | Lys | Ala | Phe | Val | Leu | Ser | Ser | Val | |
| | | | | | 235 | | | | | 240 | |
| | Asp | Glu | Leu | Glu | Gln | Gln | Arg | Asp | Glu | Ile | |
| | | | | | 245 | | | | | 250 | |
| 20 | Val | Ser | Tyr | Leu | Cys | Asp | Leu | Ala | Pro | Glu | |
| | | | | | 255 | | | | | 260 | |
| | Ala | Pro | Pro | Pro | Thr | Leu | Pro | Pro | His | | |
| | | | | | 265 | | | | | | |

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:

- | | | |
|----|-----|-------------------------------|
| 25 | (A) | LENGTH: 36 |
| | (B) | TYPE: Nucleic acid |
| | (C) | STRANDEDNESS: single stranded |
| | (D) | TOPOLOGY: Linear |

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

| | | |
|----|---|----|
| 30 | GAC AAC ATC ACC GTG GCC AAT CTG GCC GTG CTC AGG | 36 |
|----|---|----|

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34
- (B) TYPE: Nucleic acid
- (C) STRANDEDNESS: single stranded
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

TGAGGAGACG GTGACCGTGG TACCTTGGCC CCAG

34

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22
- (B) TYPE: Nucleic acid
- (C) STRANDEDNESS: single stranded
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

GTTAGATCTC CAGCTTGGTC CC

22

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22
- (B) TYPE: Nucleic acid
- (C) STRANDEDNESS: single stranded
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

AGGTSAMCT CCAGSAGTCW GG

22

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: single stranded

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

TTCTGCAGAT ATTGTGCTAA CTCAGTCTCC

30

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15

(B) TYPE: Amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
5 10Gly Gly Gly Gly Ser
15

THE CLAIMS

1. A bifunctional molecule capable of inhibiting activation of a platelet and/or adhesion of a platelet to a damaged or diseased vascular surface, said bifunctional molecule comprising two linked domains, a first domain capable of inhibiting the binding of thrombins to platelet glycoprotein Iba and a second domain capable of inhibiting the binding of von Willebrand factor to platelet glycoprotein Iba.
2. A bifunctional molecule according to Claim 1 comprising two covalently-linked domains.
3. A bifunctional molecule according to Claim 2 having a first domain selected from the group consisting of:
- (A) an antibody, or an active fragment thereof, having as its epitope, or as a part thereof, all or part of a thrombin binding site of the platelet provided by glycoprotein Iba;
 - (B) a polypeptide comprising approximately the amino terminal His¹ to Arg²⁹³ fragment of glycoprotein Iba, in derivatized or underivatized form, or one or more subfragments thereof containing all or part of the thrombin-binding site thereof;
 - (C) a mutant or derivative of a polypeptide of (B) above;
- and
- (D) an organic analog patterned on any of (A) through (C) above and having all or part of the binding activity thereof;
- and a second domain consisting of:
- (A) an antibody, or an active fragment thereof, having as its epitope, or as a part thereof, all or part of the von Willebrand factor binding site of platelet glycoprotein Iba;
 - (B) a polypeptide comprising approximately the Arg⁴⁴¹ to Val⁷³³ fragment of mature von Willebrand factor subunit, in derivatized or underivatized form, or one or more subfragments thereof containing all or part of the glycoprotein Iba binding site thereof;
 - (C) a mutant or derivative of a polypeptide of (B)

above;

and

- (D) an organic analog patterned on any of (A) through (C) above and having all or part of the binding activity thereof.

5 4. A bifunctional molecule according to Claim 3 comprising two covalently linked domains, a first domain consisting essentially of a fragment of LJ-Ib10 antibody containing all or part of the variable region thereof, and a
10 second domain consisting essentially of that fragment of mature von Willebrand factor subunit from approximately residue Arg⁴⁴¹ to approximately residue Val⁷³³ thereof, or a subfragment thereof containing residues Leu⁶⁹⁴ to Pro⁷⁰⁸.

5. A bifunctional molecule according to Claim 3
15 comprising two covalently linked domains, a first domain comprising that fragment of glycoprotein Ib α from approximately residue Gly²⁷¹ to approximately residue Glu²⁸⁵ thereof, and a second domain consisting essentially of a fragment of LJ-Ib1 antibody containing all or part of the
20 variable region thereof.

6. A bifunctional molecule according to Claim 3 comprising two covalently linked domains, a first domain comprising that fragment of glycoprotein Ib α from approximately residue Gly²⁷¹ to approximately residue Glu²⁸⁵
25 thereof, and a second domain consisting essentially of that fragment of mature von Willebrand factor subunit from approximately residue Arg⁴⁴¹ to approximately residue Val⁷³³ thereof.

7. A method of inhibiting binding of thrombins to a
30 high affinity thrombin binding site on a platelet, said binding site being located on platelet membrane glycoprotein Ib α , said binding site and thrombins having also predetermined affinities, said method comprising providing a mixture of platelets, thrombins, and also a peptide patterned
35 upon the amino acid sequence of said high affinity site of glycoprotein Ib α , wherein the platelets and thrombins of said resultant mixture have a decreased affinity relative to said predetermined affinities, and said peptide is provided at a concentration in said mixture insufficient to inhibit,

significantly, binding of thrombins to medium and low affinity thrombin binding sites of the platelets contained therein.

8. A method according to Claim 7 effective to inhibit platelet activation or aggregation in a patient.

9. A method according to Claim 7 effective to inhibit thrombosis in a patient.

10. A method according to Claim 7 wherein the peptide used therein is selected from a group consisting of Gly²⁷¹-Glu²⁸⁵, Asp²⁶⁹-Glu²⁸² and Pro²⁶⁵-Glu²⁸⁵ of glycoprotein Ib α .

11. A DNA sequence encoding a bifunctional molecule according to Claim 2.

12. An expression plasmid or viral expression vector containing an encoding DNA according to Claim 11, said plasmid or vector being capable of replication in a host cell and directing expression therein of said bifunctional molecule.

13. A recombinant host cell transformed with an expression plasmid or viral expression vector according to Claim 12.

14. A process of producing a bifunctional molecule according to Claim 2, said process comprising culturing a host organism transformed with a biologically functional expression plasmid that contains a DNA sequence encoding said bifunctional molecule under conditions which effect expression of the molecule by the host organism, and recovering said molecule therefrom.

15. A polypeptide comprising approximately the amino terminal His¹ to Arg²⁹³ fragment of glycoprotein Ib α , in derivatized or underivatized form, or one or more subfragments thereof, said polypeptide containing also a sulfate group attached to the phenolic oxygen of one or more tyrosine residues of said polypeptide.

16. A sulfated polypeptide according to Claim 15 consisting essentially of the Gly²⁷¹-Glu²⁸⁵ subfragment of platelet glycoprotein Ib α .

17. A therapeutic composition comprising one or more bifunctional molecules according to Claim 1 effective to inhibit binding of thrombins and/or von Willebrand factor to

platelets, and a pharmaceutically acceptable carrier.

18. A therapeutic composition according to Claim 17 comprising one or more additional therapeutic substances.

19. A therapeutic composition comprising one or more sulfated polypeptides according to Claim 15 effective to inhibit binding of thrombins and/or von Willebrand factor to platelets, and a pharmaceutically acceptable carrier.

20. A therapeutic composition according to Claim 19 comprising one or more additional therapeutic substances.

21. A method of inhibiting thrombosis in a patient, said method comprising administering to such patient an effective amount of a composition according to Claim 17.

22. A method of inhibiting thrombosis in a patient, said method comprising administering to such patient an effective amount of a composition according to Claim 19.

23. A bifunctional molecule capable of inhibiting activation of a cell, said bifunctional molecule comprising two linked domains, a first domain capable of inhibiting the binding of thrombins to glycoprotein Iba of a cell and a second domain capable of inhibiting the binding of von Willebrand factor to glycoprotein Iba of a cell.

24. A bifunctional molecule according to Claim 23 capable of inhibiting activation of endothelial cells or smooth muscle cells.

25. A therapeutic composition comprising one or more bifunctional molecules according to Claim 23 effective to inhibit binding of thrombins to glycoprotein Iba of a cell and/or the activation of said cell, and a pharmaceutically acceptable carrier.

26. A method of inhibiting thrombosis in a patient, said method comprising administering to such patient an effective amount of a composition according to Claim 25.

27. A method of inhibiting activation of cells in a patient, said method comprising administering to such patient an effective amount of a therapeutic composition, said composition comprising the amino terminal His¹-Arg²⁹³ fragment of GPIba, in derivatized or underivatized form, or one or more subfragments thereof, and a pharmaceutically acceptable carrier.

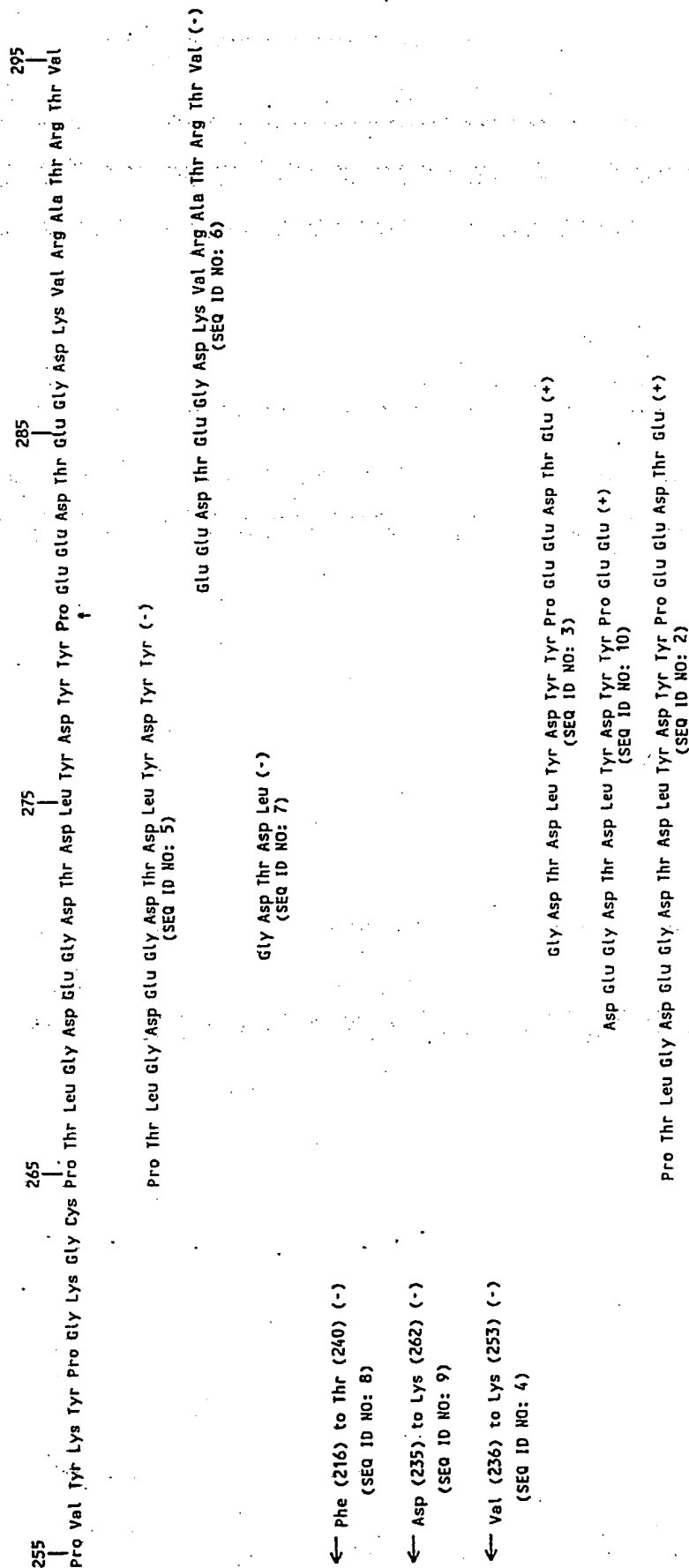


FIGURE 1

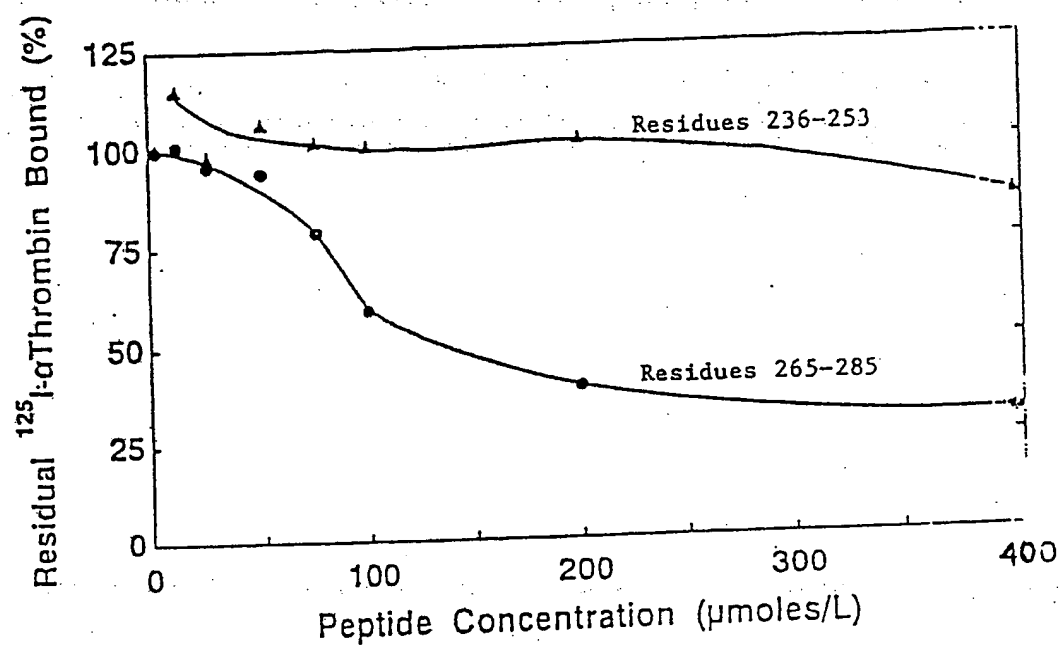


Figure 2

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/10947

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 69.1, 69.6, 172.3, 240.2, 252.3, 320.1; 514/8, 12; 530/383; 536/27; 935/10

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Medline, Biosis

search terms: GPIB, thrombin, sulfat?, thrombin binding, sulfation

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|-----------------------|
| Y | J. BIOL. CHEM., Volume 264, No. 29, issued 15 October 1989, Mohri et al., "Isolation of teh vonWillebrand Factor Domain Interacting with Platelet Glycoprotein Ib, Heparin, and Collagen and Characterization of Its Three Distinct Functional Sites," pages 17361-17367, see entire document. | 1-28 |
| Y | J. BIOL. CHEM., Volume 261, No. 27, issued 25 September 1986, Handa et al., "The von Willebrand Factor-binding Domain of Platelet Membrane Glycoprotein Ib," pages 12579-12585, see entire document. | 1-28 |

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

| | |
|---|--|
| * Special categories of cited documents: | "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" document defining the general state of the art which is not considered to be part of particular relevance | "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" earlier document published on or after the international filing date | "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "A" document member of the same patent family |
| "O" document referring to an oral disclosure, use, exhibition or other means | |
| "P" document published prior to the international filing date but later than the priority date claimed | |

Date of the actual completion of the international search

11 March 1993

Date of mailing of the international search report

7 MAR 1993

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Authorized officer

ROBERT A. WAX

Facsimile No. NOT APPLICABLE

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/10947

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|-----------------------|
| Y | J. CLIN. INVEST., Volume 86, issued July 1990, DeMarco et al., "Variant Bernard-Soulier Syndrome Type Bolzano - A Congenital Bleeding Disorder Due to a Structural and Functional Abnormality of the Platelet Glycoprotein Ib-IX Complex," pages 25-31, see entire document. | 1-28 |
| Y | US, A, 4,816,397 (Boss et al.) 28 March 1989. See entire document. | 1-28 |
| Y | BLOOD, Volume 76, No. 5, issued 01 September 1990, Hortin, "Sulfation of Tyrosine Residues in Coagulation Factor V," pages 946-952, see entire document. | 15-16 |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/10947

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (5):

C07H

A61K 35/14, 37/10; C07H 15/12, 17/00; C07K 03/00, 13/00, 15/00, 17/00; C12N 01/20, 05/00, 05/02, 15/00; C12P 21/06; C12Q 01/00

A. CLASSIFICATION OF SUBJECT MATTER:

US-CL :

435/7.1, 69.1, 69.6, 172.3, 240.2, 252.3, 320.1; 514/8, 12; 530/383; 536/27; 935/10